1968

A Study of Some Physico-Chemical Changes in a Spread-Type Dairy Product During Storage

Alfredo E. Gudeikis

A STUDY OF SOME PHYSICO-CHEMICAL CHANGES IN A SPREAD-TYPE DAIRY PRODUCT DURING STORAGE

BY

ALFREDO E. GUDEIKIS

A thesis submitted
in partial fulfillment of the requirements for the
degree Master of Science, Major in
Dairy Science, South Dakota
State University

1968

A STUDY OF SOME PHYSICO-CHEMICAL CHANGES IN A SPREAD-TYPE DAIRY PRODUCT DURING STORAGE

This thesis is approved as a creditable and independent investigation by a candidate for the degree, Master of Science, and is acceptable as meeting the thesis requirements for this degree, but without implying that the conclusions reached by the candidate are necessarily the conclusions of the major department.

Thesis Adviser — Date

Head, Dairy Science Department — Date

ACKNOWLEDGMENTS

The author wishes to express his sincere gratitude to Associate Professor Kenneth R. Spurgeon for his guidance, advice, encouragement and collaboration in this research work and thesis preparation.

Grateful recognition, too, is accorded to Mr. Shirley Seas and the workers in the Dairy Products Laboratory for assistance during the preparation of the samples for this work.

Special thanks is given also to Dr. Neal Jorgensen for his generous aid and helpful guidance in some phases of the laboratory work.

Thankful appreciation is expressed to Miss Bonnie Christie for performing or helping with many of the analyses and in helping to package the lots of spread.

Utmost gratitude is accorded to Mrs. Marlys Beukelman for her zeal, perseverance, and standards of excellence in typing this thesis and for patience with all the changes that were pressed upon her.

And to all the members of Dairy Science staff who helped in various ways in the completion of this study the warmest of thanks are expressed.

AEG

TABLE OF CONTENTS

TABLE OF CONTENTS
(Continued)

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

In the Dairy Science Department at South Dakota State University a new dairy spread product has been developed which has only 50% of the fat content of butter or margarine and 60% of the calorie value of the higher fat products. It is increased in solids content with nonfat milk solids. The purpose of developing this product was to obtain a new outlet for milk constituents; desirably it was to be one that could regain the market sales which were lost by butter and are now enjoyed by the margarines. The high price of butter compared with the substitute product has been an important factor in the displacement of the consumer's acceptance. The reduction in fat percentage to half the content of butter made it possible to cut down the ingredient costs, for fat is the most expensive constituent. At the same time spreadability over a wide temperature range which even allows one to spread the product immediately after removal from storage in a home refrigerator, an improved flavor, and an increased proteinic value are additional desirable characteristics of this new product. Recently the people who worked on its development hopefully presented the formula, processing data, and offer of technical aid to the dairy processors of South Dakota. The presentation was accompanied by comments on the qualities and possibilities of the product.

Since developmental work on the product was started, keeping quality had been closely observed and judged continuously. Tests and observations indicated the need of some changes in the composition

and/or procedures to improve and extend the period that the product could be stored and remain an acceptable food product. It had been observed that the dairy spread did tend to soften during storage. It was thought that this change in the body and texture involved some protein transformation and/or changes in their water holding capacity. It was thought, therefore, that an electrophoretic migration study of milk proteins in the dairy spread during a storage period of at least

l changes ac-

l to be con-

cetoin can

izes to 2, 3-

es flavor to

ole in the

it was ap-

n the dairy

ion of ascor-

bic acid.

Errata

Page 2, lines 12, 13 - and diacetyl conversely reduces to acetoin which then reduces to 2, 3-butylene glycol.

Protein sulfhydryl (-SH) groups are quite reactive, and contribute antioxygenic properties to milk proteins which have been heated to denaturation temperatures, as has been demonstrated by many workers. Their presence was supposed to be connected with the physico-chemical changes and to be a major factor in the oxido-reduction equilibria. They were evaluated quantitatively with a modified amperometric titration. It was hoped that determinations of the actual redox

and/or procedures to improve and extend the period that the product could be stored and remain an acceptable food product. It had been observed that the dairy spread did tend to soften during storage. It was thought that this change in the body and texture involved some protein transformation and/or changes in their water holding capacity. It was thought, therefore, that an electrophoretic migration study of milk proteins in the dairy spread during a storage period of at least 3 months would be useful to indicate whether the presumed changes actually occurred.

Variations in the redox potential have been reported to be connected with changes on the diacetyl level content since acetoin can be oxidized to diacetyl and diacetyl in turn readily oxidizes to 2, 3-butylene glycol. Of these three, only diacetyl contributes flavor to foodstuffs.

Since ascorbic acid was known to play an important role in the oxido-reduction equilibria of milk and milk derivatives, it was apparent that any study of the oxidation-reduction system in the dairy spread would of necessity involve quantitative determination of ascorbic acid.

Protein sulfhydryl (-SH) groups are quite reactive, and contribute antioxygenic properties to milk proteins which have been heated to denaturation temperatures, as has been demonstrated by many workers. Their presence was supposed to be connected with the physico-chemical changes and to be a major factor in the oxido-reduction equilibria. They were evaluated quantitatively with a modified amperometric titration. It was hoped that determinations of the actual redox

potential and quantitative determinations of two major constituents affecting the redox equilibria would shed some light on the inter-relationships between redox potential and the flavor of the dairy spread.

Since it had been evident during developmental work on the dairy spread that both the total and relative quantities of protein, fat, total solids, and moisture affected body, texture, and flavor of the product; and since the viable bacteria content had an obvious influence on keeping quality and oxygen content of the dairy spread; it was elected to follow the contents of these factors during the storage trials.

OBJECTIVES

A major endeavor involved in the development of the dairy spread was to extend its market shelf life. Indications were that it should be able to withstand a storage period of at least 90 days without undergoing perceptible adverse changes in body, texture, or flavor. However, most of the batches which had been prepared had been observed to undergo changes in physico-chemical equilibria which usually resulted in a softer body, altered flavor, and diminished water holding capacity.

Changes in proteins were suspected to be the cause of the body softening tendency and the reduced water holding capacity. Altered flavor was thought to be related to changes in pH and/or redox potential.

The objectives of this research were to study some such alterations during the storage time and, along with these changes, to determine interrelationships and effects on the body, texture, and flavor by following these factors in the respective manners indicated:

1. Oxidation-reduction potential determined initially and every 30 days until three months of storage.
2. Sulfhydryl groups content, which has been shown to be related to the heat denaturation of proteins and their antioxidant properties.
3. Diacetyl content in fresh and stored products and connections with the changes in redox potential.
4. Ascorbic acid, quantitative determination. A review of its properties had indicated that it was likely also to be

related to redox potential.

5. Protein degradation during storage as measured by starch gel and acrylamide vertical gel electrophoresis.
6. Total solids, moisture, fat, proteins, by Mojonnier method and microbiological counts by standard plate count procedure.

REVIEW OF LITERATURE

Previous Work on Dairy Spread

The development of dairy spread products, with lower fat content than butter, has been attempted by a number of persons and in a wide range of composition. However none had been successful enough to gain definite consumers' acceptance (107). The composition ranged from low to high milk fat content, from all dairy constituents to half or more non-dairy ingredients, from sweet to distinctly acid, and from high to relatively low moisture content. Reports showed that some were lacking in flavor; with some, flavors were not appealing at all; and generally the quality tended to deteriorate rapidly during storage, so that possible storage time was little more than a week.

A sour spread which contained about 5.5% total milk solids was patented in 1930 by Leopold (62). It was prepared by concentrating buttermilk and sugar and had this final composition: sugar 37.6%; milk fat, 1.15%; casein, 4.4%; albumin, 1.14%; lactose, 7.93%; lactic acid, 0.62%; ash, 1.41%; water to 100%. The sour taste of the spread was probably promoted by the cultured buttermilk.

A year later, in 1931, Parsons (76) prepared a food product by adding emulsifying salts to highly concentrated whole milk or skimmilk and heating with stirring until the product had a brown color and a roast beef odor. When mixed with cheese, fats or condiments, it was suitable for use as a spread, sandwich filling, or salad dressing.

In 1935 Grelck (36) patented a semi-solid sour spread. He started with whole milk, skimmilk or buttermilk, added a lactic starter which

developed acidity and coagulated the proteins, and heated the mix to the boiling point. This fermented coagulated milk was concentrated under vacuum to about 60% solids. In a separate patent (37) Grelck stated that the gel or product obtained might be flavored in various ways such as by the addition of cured cheese.

In 1939 Webb and Hufnagle (100) prepared a spread by mixing equal weights of sweetened condensed whey and peanut butter.

In 1943 Weckel (101) developed a spread at the University of Wisconsin. Various sources of milkfat and nonfat milk solids were used; also cultured buttermilk, lactic acid, and starter distillate were used to add flavor. This product with the name of Dyne spread was placed on the market by Madison dairies in October, 1943. Its composition was 28% fat, 19% solids-not-fat. The ingredients were blended, pasteurized at 150°F. for 30 minutes, homogenized and packaged. The product had satin-smooth texture, excellent plastic spread qualities, quite bland flavor and the perishability characteristics of cottage cheese. However, after a short time government officials ruled that its manufacture would place a drain on the low supply of butterfat and its manufacture was discontinued.

Afterward, in 1952 and again in 1965 Weckel (102, 103) reported on further formulation of a dairy spread with 40%, 45%, or 50% fat and 12%, 9%, or 6% solids-not-fat, respectively. Among the reasons for which Weckel stated that the production of dairy spreads should interest the milk distributors were: (a) utilization of butter is declining, (b) dairy spreads are easily produced in a market milk plant, (c) they contain a high percentage of solids-not-fat which is

significant from the standpoint of nutrition, and (d) they have many uses such as on bread or crackers and in sandwiches.

In 1950 Whittier and Webb (107) stated "A blend of concentrated whole milk, cream, salt, vegetable gum, acetic acid, and artificial flavor and color was produced and sold during World War II as a bread spread. It was slightly acid to the taste and had a mild milk flavor."

In 1959 Roberts (84) prepared a spread with this composition: 25% milk fat and 15% milk solids-not-fat. The solids-not-fat contained 50% sodium caseinate and 30% lactose. The finished product had a gummy body and a flavor which was described by Bullock (14) as characteristic of a weak sodium hydroxide solution. In 1958 Tobias and Tracy (95) found 40% fat and 8% solids-not-fat had the most acceptance in the finished spread. They stated that 12% solids-not-fat imparted objectional flavor to the product.

In 1966 Bullock (14) made dairy spreads with 25%, 30%, 35%, and 40% fat in the final composition. He stated that 35% and 40% fat produced a more stable emulsion. Spreads were made which contained 14, 16, 18 and 20% of calcium-reduced skimmilk powder (CRSMP). The solids-not-fat levels were adjusted to 20% by the addition of regular skimmilk powder. The test showed that with 14% CRSMP no stable emulsion was developed. At 16 and 18% the product was acceptable, so a spread with 17% CRSMP was developed. Butter or butteroil was used to fulfill fat requirements; the latter yielded softer body. The use of butteroil led to a granular texture in the finished product. About the texture Bullock stated "After two or three weeks' storage at 45°F. or lower, the spread became mealy in texture owing to crystallization

of lactose." He stated also that the shelf life could be extended by freezing because this did not destabilize the body. On the other hand, Weckel (102) and Parsons (76) recommended that the product should not be frozen but should be continuously refrigerated.

Tobias and Tracy (95) reported the use of gelatin as stabilizer at the level of 0.3% in order to stabilize the body and to prevent syneresis and wheying-off. They also reported that the addition of a mild alkali stabilized the mineral content, prevented the wheying-off, and produced a good body. Tobias and Tracy reported also that a relatively low acidity was preferred. Whittier and Webb (107) instead reported that a tart flavor was the most acceptable.

Weckel (101) reported that lactic acid produced coalescence of the fat during homogenization and a "set", congeal or "structure" upon cooling. According to his recommendations, lactic acid should be added before the homogenization process and below 100°F. temperature of the mixture. The recommended homogenization pressures were from 1500 to 2500 pounds per square inch for single stage. The use of higher pressures resulted in a grainy or rough texture in the finished product. If two stage homogenization were used, a better bodied product was obtained with the lower pressure on the first stage. Tobias and Tracy (95), on the contrary, recommended 3500 pounds per square inch pressure for a single stage homogenization.

Wilster (108) reported that in order to thicken slightly the spread should be homogenized at sufficient pressure. Roberts instead (84) stated that "Homogenization was not required to form a stable emulsion. This was due in part to the water binding capacity of

sodium caseinate."

Brunner (15) reported that homogenization disperses evenly the fat globules, increases their number, and narrows the range of their size, which stabilizes them against gravity separation. Today homogenization is a standard method used in the dairy industry. It more noticeably effects an increase of viscosity in products of higher fat content.

Oxidation-Reduction Potential of Milk

Jenness, Shipe and Whitnah (52) stated that in the case of organic materials, oxidation may be defined as the uptake of oxygen or the loss of hydrogen. In the same manner reduction may be defined as the process of losing oxygen or gaining hydrogen. In a broader sense the processes of oxidation and reduction are not necessarily limited to the gain or loss of oxygen and hydrogen. In ionic systems the phenomenon involves the loss or gain of electrons. Oxidation is the loss of electrons and reduction is the gain of electrons. Every oxidation is accompanied by a reduction, and vice versa. In the chemistry field, compounds are usually characterized as oxidizing or reducing agents. The relative tendency of a substance in solution to yield or take on electrons can be measured if a standard substance that will donate or accept electrons can be inserted in the system to establish a difference in potential. Practically, the difference in potential created by a platinum electrode in a solution of an oxidant or reductant is measured by closing a circuit through a calomel half-cell and a potentiometer. The voltage measured in this circuit represents the oxidizing or reducing capacity of the solution. This potential is known as the

oxidation-reduction potential or the redox potential and it is designated by the symbol Eh. Positive potential indicates oxidizing properties of loss of electrons; and negative, involving gain of electrons at the platinum electrode, reveals reducing capacity.

From the principles cited before and the law of mass action, the following basic equation has been derived (70)

$$Eh = Eo - \frac{RT}{rF} \ln \frac{[Red]}{[Ox]}$$

where Eh = oxidation - reduction potential; Eo = standard oxidation - reduction potential of the systems; R = gas constant; T = absolute temperature; r = number of electrons transferred per molecule; F = Faraday constant; [Red] = concentration of reduced form; and [Ox] = concentration of oxidized form.

At 25°C and with one electron transfer the equation becomes: (52)

$$Eh = Eo + 0.06 \log \frac{[Ox]}{[Red]}$$

Then Eo would be the same value as Eh at equal concentrations of the oxidized and reduced forms, since the log of 1 is zero.

The most important thing in considering the oxidation-reduction potential equilibria in milk products is the potentials of the systems present relative to one another and those that may be superimposed (52).

Milk contains several oxidation-reduction systems. The influence of each on the potential depends on various factors, such as the reversibility of the system, the ratio of oxidant to reductant, and the concentration of the active components of the system. An irreversible system or "one way" system is one which is able to be oxidized or to be reduced, but not both (52). The reversible systems only give a

potential at a noble metal electrode, and this measured potential is an intensity factor similar to the potential measured on a hydrogen electrode in determining H-ion concentrations (52). In the same manner as there is buffer action in acid-base equilibria, so there is an action known as poising in oxidation-reduction equilibria. This resistance to change of potential has its greatest intensity when the concentration of reductant is equal to that of oxidant, that is when Eh = Eo (52).

Ordinary fresh raw milk has a potential at a gold or platinum electrode of between +0.20 and +0.30 volts. Dissolved oxygen is a major factor in the potential value. If milk is drawn anaerobically from the udder it will reduce methylene blue solution, because its potential is more negative than that of a methylene blue system (47). The potential of the milk rises when it is exposed to oxygen and reaches a positive potential equal to that of a methylene blue system. The potential may be changed in the negative direction if the milk containing oxygen is washed with oxygen-free gas or if Streptococcus lactis are allowed to grow (30, 41). Bubbling oxygen or air through these milks will restore the positive potential. Oxygen will induce on fresh raw milk a potential more positive than +0.02 volts. Other components of milk that may affect its potential must belong, therefore, to systems having normal potentials more negative than +0.02 volts. The most abundant components of milk (fat, sugar, and protein) do not have any influence on its oxidation-reduction potential (22). Jenness, Shipe, and Whitnah (52) stated that the actual concentrations of cysteine and glutathione in milk do not reduce methylene blue. Consequently

in oxygen-free milk the only systems that may be responsible for the oxidation-reduction potential value are ascorbate, lactate, and riboflavin and they may have an influence in the stabilization of the potential of oxygen-containing milk. The ascorbate system is reversible; the regular amount of ascorbic acid in milk is enough to impart an appreciable effect on the potential. The oxidant of the system is dehydroascorbate; it undergoes further oxidation, readily but irreversibly, so that the ratio of concentration of ascorbate to that of dehydroascorbate remains large until the system disappears from the milk. This system tends to stabilize the potential at approximately 0.0 volts.

The lactate-pyruvate system is irreversible. It is activated by the action of enzymes and mediators and is of a highly negative normal potential (5), but the total amount of this system is very small; hence, even if it is activated, the influence on the potential is very slight.

The riboflavin system is an active reversible one and of highly negative normal potential; but also its concentration in fresh milk is very small; and in the oxidized form, its influence is very slight.

Jenness, Shipe and Whitnah (52) stated "It seems logical to conclude that the ascorbate-dehydroascorbate system is the principal one stabilizing the potential of oxygen-free milk at a value near 0.0 volts and is the system that functions along with the oxygen system to stabilize the potential of oxygen-containing milk in the zone of +0.20 to +0.30 volts."

Milk suffering fermentation by Streptococcus lactis will change

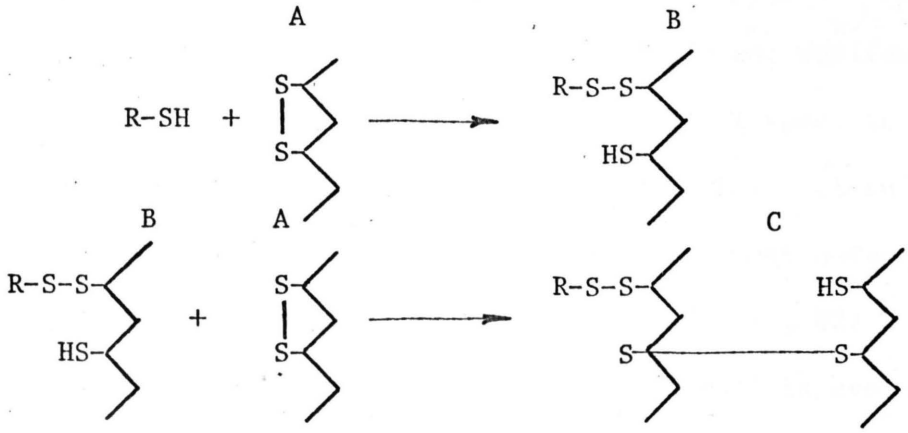
in oxidation-reduction potential, changing with time to negative values (34). A rapid change in the potential occurs after the dissolved oxygen has been consumed by the bacteria and may be identified by the change in color of certain dyes added to the milk. This "reduction time" required for the change to become manifest has come to be used as an index of the degree of bacterial contamination.

Oxidation-reduction potential is affected by the heat treatment of the milk. This phenomenon has been studied considerably (30, 41, 71). The potential decreased sharply when the sulfhydryl groups were liberated by denaturation of the proteins, primarily the β-lactoglobulin. Deaeration and high-temperature short-time heat treatments promoted minimum potentials. Such treatments produced also dried milk of improved stability against oxidative flavor deterioration (41).

Protein Sulfhydryl Groups

Proteins are formed by a large number of repeating units of L-α-amino acids, which are joined one to the other by peptide bonds. Jenness and Patton (50) listed 21 known amino acids which are usually found as constituents of proteins. Some are limited in distribution but may be present in high concentrations in a few proteins. Among these 21 amino acids there are three which contain sulfur in their composition: cysteine, cystine, and methionine. Peptides and proteins containing intramolecular disulfide linkages form aggregates linked by intermolecular disulfide bonds (-S-S). These aggregates (20) are formed in a chain reaction initiated by a substance containing free sulfhydryl groups (-SH), such as glutathione or another protein or

peptide. Diagramatically the process has been illustrated as follows (104).



R-SH reacts with the peptide chain A, which contains an intramolecular -S-S- group to form B, containing a free -SH group. B reacts then with another A to produce C containing two groups (also R-S-S-) and since C contains a free -SH group, it in turn may react with A to form an aggregate with three A groups, etc.

The denaturation of proteins appears often to involve reactions of such aggregations, according to Jenness and Patton (50) who cited the reactivity of the sulfhydryl groups in milk serum proteins when the latter are affected by heat treatment. The sulfhydryl groups are apparently occluded in or bonded to the coil configuration in the native protein so they are relatively unreactive, but when the protein is subjected to sufficient heat treatment the primitive helix starts uncoiling and the groups become more accessible and reactive. These free sulfhydryl groups may be readily oxidized, so they constitute powerful reducing agents. Hence they contribute to the antioxygenic properties of heated milk and the lowering of the oxidation-reduction potential. The activation of the sulfhydryl groups by heat treatment as measured by the nitroprusside test becomes evident

about 75°C (53). The principal site of the sulfhydryl groups in the milk serum proteins is cysteine (50).

According to Tumerman and Webb (98) an important manifestation of protein denaturation is the increased reactivity of specific groups resulting from structural modification of the protein molecule. The activation of the sulfhydryl groups is one of the most detectable of the chemical changes accompanying denaturation (3, 71, 82).

The activation of the -SH groups closely parallels evolution of volatile sulfides and the appearance of cooked flavor in milk (51). Hydrogen sulfide seems partly responsible for the cooked flavor and it is notable that the amino acid cysteine, the principal site of -SH groups in milk serum proteins, liberates H_2S when heated in aqueous solution. Factors affecting the quantity of sulfides produced in heated milk were studied by Townley and Gould (97) who found that oxidizing conditions generally inhibited and reducing conditions favored sulfide formation. When heat treatment was prolonged or extended above 75°C, cooked flavor and volatile sulfides increased in importance and the sulfhydryl groups were decreased in quantity and reducing capacity (56, 58). A number of oxidizing reagents, including o-iodosobenzoate, iodine, thiamine disulfide, iodoacetate, p-chloromercuribenzoate, ferricyanide, tetrathionate and porphyrindin have been introduced for the quantitative estimation of sulfhydryl activity in denatured proteins. Their application to studies on the milk serum proteins has been studied by Jenness (48) and Coulter et al. (25). The dominant sulfhydryl bearing component of the milk serum proteins, β-lactoglobulin, possesses an estimated four -SH groups per molecule on

the basis of a cystine equivalent of 1.3% and a molecular weight of 36,000 (46, 58). Sulfhydryl group activation by heat has been associated with the origin of cooked flavor as well as the antioxygenic properties of heated milk (26, 41, 109). The oxidations apparently result in the oxidation of two sulfhydryl groups to a disulfide group. Presumably such an oxidation may involve two sulfhydryl groups in the same peptide chain or in different peptide chains, which would cause linkage of the chains and formation of a different and more complex protein structure (104).

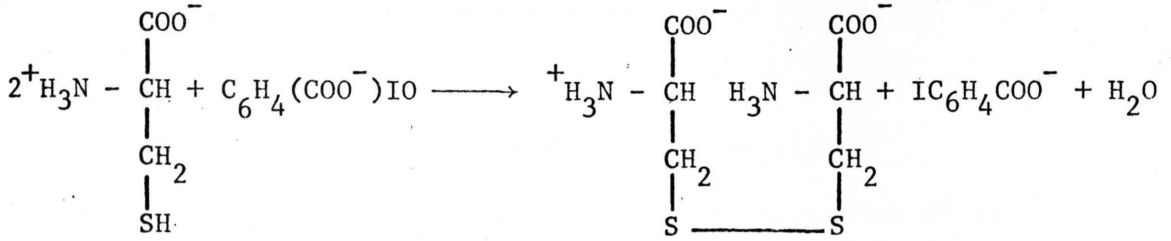
Hess and Sullivan (45) found that the amount of iodine reduced by native proteins in acid solutions corresponded to their cysteine content as determined by colorimetric analysis of acid hydrolyzates.

It has been studied and clearly established that there are several degrees of reactivity or availability of the sulfhydryl groups of proteins. Anson (2), reported that iodine reacted with all the sulfhydryl groups of native egg albumin and iodoacetamide with about the half of them, but that the reagent nitroprusside showed a negative test.

The simple iodoso compounds, functioning as powerful oxidizing agents, ordinarily are reduced practically irreversibly. This characteristic and the stability observed in some studies of a slightly alkaline solution of certain salts of the readily available o-iodosobenzoic acid, suggested a new approach to the troublesome problem of the estimation of cysteine and certain of its derivatives (44).

This approach is based upon a reaction which has been found to

proceed under proper conditions according to the following formula:



O-iodosobenzoate was first proposed by Hellerman (44) as an oxidant for the quantitative determination of protein sulfhydryl groups. Hellerman, Chinard and Deitz (43) determined by inhibition tests that the sulfhydryl groups of the enzyme urease were partially oxidized, and also that cysteine, glutathione, and apparently sulfhydryl groups of guanidine-denatured proteins were quantitatively oxidized to the respective disulfide compounds. In previous tests with egg albumin and β-lactoglobulin the o-iodosobenzoate method yielded the same titration values for the native and for the guanidine-denatured protein.

Larson and Jenness (59) applied the o-iodosobenzoate method to a kinetic study of the heat activation of the sulfhydryl groups in crystalline β-lactoglobulin. In the temperature range 64-75°C the sulfhydryl groups were activated according to a first order reaction with an activation energy of 80,000 cal/mole. This activation of sulfhydryl groups seemed to parallel the primary denaturation phase (12). Larson and Jenness (57) affirmed that "The fact that iodine apparently reacts with all the sulfhydryl groups of native proteins and o-iodosobenzoate with only the more reactive or accessible ones such as are present in guanidine-denatured egg albumin (44) suggested that in titrating proteins by the o-iodosobenzoate method

part of the oxidation may be due to iodine, since the excess o-iodosobenzoate is determined by liberation of iodine from iodide ion in acid medium. Apparently the stoichiometry of the oxidation, whether produced by iodine or o-iodosobenzoate, is similar".

Larson and Jenness used an amperometric adaptation of the procedure usually used for the "dead point" titration (33, 94). The amperometric "dead point" titration has been found to be very useful for the titration of opaque solutions which hide the color of the free iodine when it appears. Starch indicator did not indicate exactly the end point either because it did not form a visible complex with iodine until the iodine normally reached 1 to 10 x 10^{-6}N depending on the concentration of the iodine and the type of starch used. Therefore by reading a slight permanent deflection of the galvanometer it was possible to find the end point of the titration by extrapolation to zero current flow which gave the dead point on plotting of the reading of the galvanometer against the milliliters of o-iodosobenzoate added. Also, Larson and Jenness (58) studied the reducing capacity of milk as measured by an iodimetric titration and demonstrated that the o-iodosobenzoate titration method as modified by themselves (57) was applicable to milk and other dairy products, which made it possible to use the method with opaque solutions such as milk or milk products. This method was modified slightly and used for sulfhydryl titrations in the present study. Larson and Jenness (58) also studied the effect of higher temperature heat treatments on the o-iodosobenzoate reducing capacity of milk, milk serum proteins, and crystalline β-lactoglobulin and showed how the

reducing capacity as cysteine of the samples was reduced progressively when samples were heated for 30 minutes at temperatures ranging from 64° to 97°C. These data confirmed the previous report (56) that heat treatment decreased the sulfhydryl reducing capacity of milk serum proteins. Probably this change was due to oxidation since it was largely prevented by excluding air from the sample during heating.

Josephson and Doan (53) and Townley and Gould (96) reported that materials in the fat phase (but not the fat itself) reduced o-iodosobenzoate and/or iodine; for instance, the materials adsorbed on the fat globule were a source of heat labile sulfides in milk. While it is probable that the principal dialyzable reductant was ascorbic acid, no claim could be made that it was the only one. Milk dialyzate titrated with o-iodosobenzoate gave approximately the same value as did the titration with 2, 6-dichlorophenolindophenol; but it was recognized that neither method was specific for ascorbic acid. Larson and Jenness (57) reported also considerable variability in the sulfhydryl content of the serum protein fraction of various samples of milk and that β-lactoglobulin was undoubtedly the principal contributor to the reducing power of this fraction, since crystalline preparations reduced 0.104 to 0.110 m.eq. of iodine per gram. Larson and Jenness (57) stated: "Thus if β-lactoglobulin represented 50% of the proteins of milk serum proteins it alone would account for titration values in the range obtained for the latter. Electrophoretic analyses indicate that components having the mobility of β-lactoglobulin comprise at least 50% of the proteins of

milk serum (28, 90). β-Lactoglobulin constitutes the major portion of the classical "lactalbumin" fraction. It is not known definitely whether the variations in sulfhydryl content of the various serum protein samples reflect the relative amount of β-lactoglobulin present, although this is strongly suspected of being the case". After milk serum proteins, β-lactoglobulin, and skimmilk had been heated separately, it was found that the decreases in reducing capacity were similar. This indicated again that β-lactoglobulin was probably the principal constituent involved (57).

Other methods for determination of sulfhydryl groups include: a polarographic method in which a reagent for sulfhydryl groups, such as silver ion, is added until it is in excess, when a diffusion current flows and can be detected at appropriate voltages (7). In a spectrophotometric method which uses p-chloromercuribenzoate, mercaptide formation is accompanied by increase in absorbancy at 250 mμ (9). Other methods were reviewed and published by Light and Smith (63).

Diacetyl in Dairy Spread

The compound diacetyl or biacetyl which chemically is the diketone 2, 3-butanedione is the most important flavor constituent of butter and butter cultures. It does not occur naturally in milk fat, but it can be obtained from bacterial fermentation of milk and/or the cream before churning. During the 1930's commercial lactic cultures were used which were incorporated into the cream before the churning process (69, 80).

The paramount role of diacetyl in providing the desirable aroma has been studied by many investigators. Among the pioneers, in 1929, van Niel, Kluyver and Derx (99) found from 2 to 4 parts per million diacetyl in fine butter; and when these amounts of diacetyl were added to a butter neutral in odor a characteristic aroma appeared. They concluded that diacetyl is either responsible for the aroma of butter or is the principal component of the aroma material. The diacetyl was supposed to come from acetylmethylcarbinol. Margarine to which diacetyl had been added was characterized by the aroma of butter (99).

The investigations continued on the production of diacetyl and acetylmethylcarbinol in butter cultures. Michaelian, Farmer and Hammer (68) reported:

"a. Butter cultures with a satisfactory flavor and aroma contained considerable quantities of acetylmethylcarbinol plus diacetyl, while cultures lacking in flavor and aroma contained small amounts or none. From a quantitative standpoint the carbinol was much more important than the diacetyl.

b. During the early stages of the ripening of satisfactory butter cultures only small amounts of acetylmethylcarbinol plus diacetyl were present, while late in the ripening conspicuous increases in the materials occurred.

c. Milk cultures of Steptococcus lactis or the citric acid fermenting streptococci normally present in butter cultures contained little or no acetylmethylcarbinol plus diacetyl, but the production of these compounds by the latter type was strikingly increased by

the addition of the proper amount of any one of a number of acids; it accordingly appears that the citric acid fermenting streptococci produce conspicuous amounts of acetylmethylcarbinol plus diacetyl only in the presence of considerable acid.

d. The source of the acetylmethylcarbinol plus diacetyl produced in a butter culture is evidently citric acid."

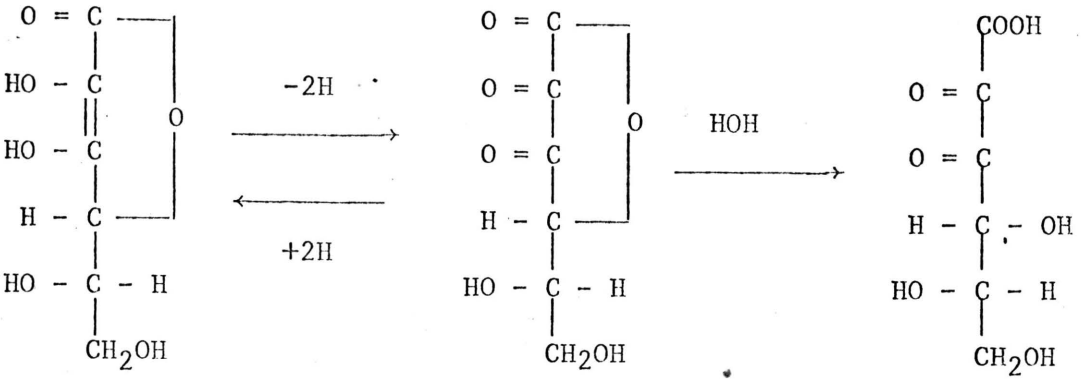
Two types of bacteria are necessary to produce a good butter culture: Leuconostoc citrovorum and Leuconostoc dextranicum, which are citrate fermenters (1). They produce diacetyl, acetylmethylcarbinol, 2, 3-butyleneglycol, volatile acids such as propionic and citric, and carbon dioxide. The main flavor constituent of the culture is diacetyl. All are produced from citric acid and citrate which are transformed into lactic acid and diacetyl.

Today it is a common practice to add flavoring material to butter in two ways: as natural culture or as culture distillate known as "starter distillate". The latter is the more common practice because no ripening time is necessary to develop diacetyl. The samples prepared for this research were prepared with starter distillate plus a synthetic flavoring material containing diacetyl, lactic acid, and other flavor compounds.

Ascorbic Acid

Ascorbic acid or vitamin C is a white crystalline substance very soluble in water and insoluble in most organic solvents. The compound exists as both the D- and L-isomers but only the L-isomer is biologically active.

The strong reducing property of vitamin C depends on the loss of hydrogen atoms from the hydroxyls on the double bonded (enediol) carbons. This oxidation of ascorbic acid yields dehydroascorbic acid. This is a freely reversible reaction. The dehydro form, except in rather acid solution, undergoes hydrolysis at the lactone ring with the formation of diketogulonic acid.



L-ascorbic acid Dehydro-L-ascorbic acid L-diketogulonic acid

Milk freshly secreted from the udder contains vitamin C only in the form of L-ascorbic acid. This reduced form of the vitamin is slowly converted to the reversibly oxidized but still biologically active form, dehydroascorbic acid, which in turn is further oxidized irreversibly to the biologically inactive diketogulonic acid.

The greater stability of ascorbic acid in acid solution depends on the decreased tendency toward hydrolysis of the lactone ring with decreasing pH. In alkaline solution the hydrolysis is fairly rapid. Its destruction is catalyzed by light, particularly in the presence of riboflavin.

Vitamin C is synthesized in the cow's body in the microsomes of the liver and possibly in intestinal and kidney tissues also. Various hexose sugars such as fructose, mannose, glucose, and glactose can

serve as precursors.

Average values of fresh raw milk (42) show that milk contains more than 20 mg. of vitamin C per liter. However for market milk the averages computed are about half of this value. The loses in vitamin C takes place at many stages in processing and distributing milk.

Relation of vitamin C content to oxidation-reduction potential of milk has been studied by Campbell, Phelps, and Keur (18) and an inverse relation was reported. Moreover, the oxidation-reduction potential determined the amount of oxidation that took place. A low E_h almost completely inhibited the oxidation of vitamin C, while a high potential rapidly destroyed the vitamin.

Determination of Ascorbic Acid (105)

Two principles have been used commonly in the quantitative determination methods for vitamin C:

1. The strong and rapid reducing property of ascorbic acid can be determined by titration against a standard oxidizing solution. Currently the dye 2, 6-dichlorophenolindophenol (2, 6-dichlorobenzenoneindophenol) is the preferred oxidant. The oxidized form, dehydroascorbic acid, does not reduce the above dye. Treatment of the acid extract with H_2S will convert the oxidized to the reduced form (29). Since both the oxidized and the reduced form of ascorbic acid have vitamin activity, it is essential that this conversion be made when the nutritional value of the product is being measured.

Many new approaches have been used in analyzing for ascorbic acid by titration procedures:

a. A definite amount of ascorbic acid is titrated with dye until pink color appears and remains at least for several minutes.

b. A definite amount of dye is titrated with the ascorbic acid solution until the pink color disappears.

c. A known excess amount of the dye solution is added to the ascorbic acid solution and the decrease in intensity of color due to reduction of the dye is determined by use of a photoelectric colorimeter.

2. The second chemical principle used in the quantitative determination of ascorbic acid involves the conversion of the vitamin into a soluble colored complex by treatment with 2, 4-dinitrophenylhydrazine and colorimetric determination of the intensity of the color.

A critical review of various methods can be found in the reports of Roe (85) and Mapson (66).

Electrophoresis Determination of Protein Fractions

Electrophoresis developed by Tiselius in 1937 was based on the principle that in a mixture of proteins each protein with its characteristic surface electric charge will respond to an applied electrical potential in a different and characteristic manner at a given pH. An account of electrophoretic analysis a bit over two decades later was provided by Cooper (24). Recent papers by Bloemendahl (8) and by Bailey (4) gave an abundance of experimental detail.

Formerly, proteins were classified largely according to their solubility. Albumins were defined as proteins soluble in water and dilute salt solutions (49). The term globulin was used for proteins insoluble in water but soluble in dilute salt solutions. Globulins were precipitated by saturation with $MgSO_4$ or half saturation with $(NH_4)_2SO_4$. However, modern techniques have shown that this classification was not justifiable since many of the preparations that have been considered individual proteins are really mixtures and exhibit gradations from one category to another.

Casein is defined as the protein precipitated by acidifying skimmilk to a pH value near 4.6. The proteins remaining after the removal of the casein are known as whey proteins or milk serum proteins (49). By salting out methods they have been fractioned to produce the lactalbumin fraction and the lactoglobulin fraction (27, 73). The casein fraction, the lactalbumin fraction, and the lactoglobulin fraction were considered as single chemical entities until Linderstrom-Lang (64, 65) in 1925, Cherbuliez and Schneider (21) in 1932, and Bugai (13) in 1935 reported the fractionation of casein and Palmer (75) in 1934 reported further fractionation of the whey proteins. Since then research has resulted in the isolation of eight main components of milk proteins, several of which are apparently heterogeneous as indicated by electrophoretic measurements. These eight components may be defined by their isoelectric points and by their electrophoretic mobilities (35).

Starch Gel Vertical Electrophoresis.

According to Boyer (10) a specific description of starch granule

and starch gel electrophoresis was given by Fine and Costello in 1963 (31). Smithies (91, 92, 93) first developed electrophoresis in starch gel. This method constituted a considerable advance over other forms of zone electrophoresis. Electrophoretic separations in media such as paper or starch granules, e.g., starch block of Kunkel (54) and Kunkel and Slater (55) depended principally on the electrostatic charges of materials examined. An additional advantage for separation was obtained by working with starch gel because the variable penetrable pores of the gel resulted in molecular sieving and this permitted small molecules to migrate through the gel more easily than large molecules. Therefore electrophoretic separation of a given sample in starch gel depended both on electrostatic charge as in the methods above and upon the molecular size. Another additional factor, especially in nonaqueous media, was the adsorption of materials to the media employed. This "chromatographic effect" in starch gel has not been studied extensively yet.

Another advantage of starch gel over other media is the simple and easy way the samples can be inserted and the comparative freedom from boundary effects and attendant smearing of analysis in the substance of the gel. The efficacy of starch gel electrophoresis is easily demonstrated by analysis of human serum proteins where no overlapped components are obtained (10).

Starch gel electrophoresis was orginally performed in horizontal molds (91). In order to avoid electrodecantation of the samples, these were inserted into the gel on a vehicle such as a bit of paper or starch granules. Smithies (93) improved this condition by using a

vertical gel into which the sample could be directly inserted as a liquid. The vertical system permitted use of larger amounts of samples without sacrifice of resolution and generally resulted in a more reproducible analysis.

Polyacrylamide Gel Vertical Electrophoresis.

Pallavicini (74) reported that column electrophoresis apparatuses were developed in Swedish laboratories in the 1950's (19, 32, 39, 77, 78). However, gels could not be used in those because of the difficulty in the recovery of the intact columns after electrophoresis or the elution of the electrophoretically separated zones with solvent flow. Gels were used in slabs or blocks as in Smithies starch gel electrophoresis (91) or as gel - slurry. New possibilities were introduced with the use of discontinuous buffers (79) and acrylamide gel as a supporting medium (72, 83). Ornstein and Davis (72) combined these with success and obtained excellent resolution. Some of the advantages of the polyacrylamide gels, which make them an ideal substrate for electrophoresis (74) are:

-Adaptable to special demands by increasing or decreasing their pore size

-Clearness

-Chemical inertness

-Physical strength

-Lack of charged groups

-Variety of staining procedures possible

-Quantitative recovery from the zones

However two disadvantages exist:

1. The pore size of the gels may be altered unintentionally by increasing or decreasing the acrylamide concentration. Large pore size-low concentration gels are fragile and difficult to handle; concentrations of 5-7% or higher have small pore size and large molecules cannot penetrate the gel.
2. The gel is toxic; therefore, caution is urged in its use.

The increasing number of publications in which the use of polyacrylamide gel as an electrophoretic supporting medium was described has demonstrated clearly its usefulness in biological research (109).

EXPERIMENTAL PROCEDURE

Oxidation-Reduction Potential Determination

Oxidation-reduction potential measurements with metallic electrodes constitute the basis of numerous chemical analyses which are important in the control of many industrial processes and useful in the study and control of biological systems.

Significant improvements in potentiometers and electrodes have resulted in greater accuracy and convenience in making oxidation-reduction potential measurements. Metal electrodes have been developed which are relatively free from polarization effects and have better reproducibility.

Oxidation-reduction potentials usually have been expressed in millivolts. This potential as measured with a pH meter (which was set to read in millivolts as the Beckman Model GS pH meter) (6) was the electro-motive-force (EMF) difference developed between a constant voltage reference electrode, e.g., saturated calomel, and a metallic indicator electrode when these electrodes were immersed in the solution under test. The nature of the solution under test and the method to be used determined the choice of the electrode. It has been found that three different types of metallic electrodes have been used: (6)

1. First type of metallic electrode.

   Consisted of a metal in contact with a solution of its own ions, e.g., silver in a silver nitrate solution. They could be used for the direct measurement and potentiometric titra-

tion of a solution of the cation of the particular metal electrode being used.

2. Second type of metallic electrode

   Consisted of a metal coated with a sparingly soluble salt of this metal in contact with a solution of a soluble salt containing the same anion, e.g., silver-silver chloride in a solution of potassium chloride. Electrodes of this type were used for the direct determination or potentiometric titration of a solution containing the anions of the sparingly soluble salt forming the coating on the electrode.

3. Third type of metallic electrode.

   Consisted of an unattackable metal such as gold, platinum or rhodium in contact with a solution containing both the oxidized and reduced states of an oxidation-reduction system, e.g., platinum in contact with a solution of ferric-ferrous ions. These electrodes were used for potentiometric oxidation-reduction titrations, and for the direct measurement of the oxidizing or reducing intensity of solutions.

All these three types of metallic electrodes were based on the same principle that an oxidized and a reduced state must be always present. The potential of all these types could then be expressed by a general form of the Nerst equation: (52)

$$Eh = Eo + 0.06 \log \frac{[Ox]}{[Red]}$$

Where Eh was the oxidation-reduction potential, measured as the voltage difference between the oxidation-reduction electrode and the normal

hydrogen electrode, the potential of which is zero by definition. Since the normal hydrogen electrode rarely has been used as the reference electrode in actual measurements, the measured potential (E) would not be equal to E_h. E_h could be calculated by adding algebraically the measured voltage E and the voltage of the constant voltage reference electrode used

$$E_h = E + \text{Voltage of reference electrode}$$

If the saturated calomel electrode was used as the reference electrode, the voltage of reference was 244.3 mV at 25°C, then (6)

$$E_h = E + 244.3$$

E and E_h were affected by changes in temperature. This effect was small but it had to be considered if the temperature was much above or below 25°C. The correction would normally be less than 1 mV per degree centigrade. This value varied with the electrode being used, but it was fixed for any particular electrode.

The E value of any particular oxidation-reduction system depended on all the constants of the system (E_o, n, and the voltage of the reference electrode being used to complete the cell) and on the concentrations of the oxidant and the reductant phases. Consequently, the measured value E or the calculated E_h was a function of the concentrations of the oxidant and the reductant. No matter what was the initial magnitude of the measured oxidation-reduction potential, E or E_h both would become more positive when the concentration of the oxidized form relative to the reduced form increased (i.e. oxidizing intensity became greater); on the other hand the values of E and E_h would be more negative when the concentration of the reductant in-

creased relative to the oxidant (reducing intensity became greater) (6).

The third type of electrode (unattackable metal in an oxidation-reduction system) has become the preferred one for the detection of the equivalence point in potentiometric oxidation-reduction titrations (6). This method of analysis requires the determination of the reduced agent by titration with an oxidizing agent of known concentration, or vice versa. This type of reaction is usually characterized by a large change in the E_h or E of the solution at the equivalence point. Because of this, potentiometric oxidation-reduction titrations are among the most accurate methods of chemical analyses. In this study of dairy spread oxidation-reduction potential, and determination of the protein sulfhydryl groups, this latter method was used.

A Beckman Model G pH meter and special Beckman electrodes (described below) were used in this research work for the determination of oxidation-reduction potential of the dairy spread samples. With this equipment, accuracy and reproducibility were obtained to within 0.001 volt unit. A null type meter system assured accuracy independent of the mechanical properties of a registering meter and of changes in the amplifier. Accuracy was also further assured because the maximum sensitivity of the amplifier occurred at the balance point.

With Beckman Model G pH meter measurements could be made in two ways:

a. Inside the electrostatically shielded compartment by using the small electrodes and sample beaker mounted on the door, or:

b. External to the meter using larger, internally shielded electrodes.

The second method was used with the following external electrodes, according to the description in Bulletin 86-R of Beckman pH electrodes; Metallic electrode No. 39273 with platinum inlay, 5 inches length, 30 inches connection lead. The glass-to-metal seal between platinum disc and glass body offered maximum resistance to chemical attack. Temperature range: -5 to 100°C.

The metallic electrodes were designed for use in combination with reference electrodes for measurements of oxidation-reduction potentials and electrochemical measurements employing pH meters and other electroanalytical instruments. These electrodes featured large areas of metal that provided more rapid electrode response and increased thermal stability. The platinum electrodes were especially resistant to chemical attack.

Reference electrode No. 40463. Sleeve junction calomel type, 5 inches length and 30 inches connecting lead. It had a liquid junction hole in immersion tip covered by a ground glass sleeve. Temperature range: -5 to 90°C. The purpose of the reference electrode was to permit the measurement of the potential developed at the surface of the glass or metallic electrode. The calomel-(mercury-mercurous-chloride)-internal element was surrounded by an electrolyte, normally a potassium chloride saturated solution. This solution contacted the internal element to form a conductive bridge between the calomel and the test solution in which the electrode was immersed. Electrical communication between electrolyte and test solutions was accomplished by a flow of the electrolyte solution from the electrode through a hole in the immersion tip. A tapered ground

glass sleeve fit over the immersion tip of the electrode and the electrolyte solution emerged around the end of the sleeve. This permitted a greater flow rate and also provided a larger contact area of the electrolyte solution than was normally possible with other types of reference electrodes.

Sleeve type electrodes were characterized by their high measurement accuracy enhanced by a stable liquid junction potential, allowing electrical resistance for measurements of viscous and highly saturated samples. Under conditions where another type of junction might become clogged, the sleeve type could be flushed easily.

Connecting the Electrodes

The platinum electrode was connected by the 30 inches lead to the upper pin jack in the compartment of the Beckman pH meter. The elastic band covering the hole of the immersion tip of the reference electrode was removed and the glass sleeve slid into place over the hole. The rubber sleeve covering the filling hole also was slid down exposing the filling hole to atmospheric pressure in order to assure a uniform flow of the electrolyte solution. Before beginning a series of measurements the level of the electrolyte solution was checked and replenished whenever necessary and any trapped air bubble was shaken out. The immersion tip and glass sleeve were cleaned carefully by washing off the encrustation of crystals. The terminal of the 30 inches lead was connected to the lower pin jack in the compartment. In accordance with the Beckman Instruments instructions sheet 296C, the measurements were conducted with only the lower part of the glass sleeve immersed in order to obtain the maximum stability.

Testing the Reference Electrode:

The accuracy of the sleeve type reference electrode was checked before use. It was connected to the Beckman model G pH meter in the usual manner and a new calomel reference electrode known to be good was plugged into the glass electrode jack. The scale of the pH meter was set to read in millivolts. With this pair of reference electrodes, readings were taken in a buffer solution and in different samples. The readings were identical and approximately zero with a $\pm$ 5 millivolt tolerance according to the instructions, and remained stable during 5 to 10 minutes.

Measuring Oxidation-Reduction Potentials.

With the platinum and reference electrodes connected to the respective terminals of the Beckman pH meter, the measurements were performed by these sequential steps.

1. The electrodes were immersed in the sample.
2. The range switch was turned to +mV (samples with positive polarity).
3. The meter was balanced, the pushbutton locked down and the pH dial rotated until null meter needle read zero. The reading on the mV dial indicated the potential. Each small division of the scale represented 10 millivolts. The digits readings were estimated when the needle lay between two ten-unit points.

Protein Sulfhydryl Groups.

The sulfhydryl groups were measured by a modification of the amperometric adaptation (57) of the "dead stop" titration of Foulk and

Bawden (33). Larson and Jenness (57) stated that this method depends upon the depolarizing action of iodine on a polarized platinum cathode, and that the method is especially applicable for the determination of small amounts of iodine in opaque sols such as dairy products. The apparatus employed is shown in Figure 1. It consisted of:

a. Beckman 2000 Model G pH Meter, which with a suitable pair of electrodes could be used to measure oxidation-reduction potentials. The electrodes used were:

   Metallic electrode: Beckman No. 39273, platinum. The same one used for oxidation-reduction potential

   Reference electrode: Beckman No. 40463, sleeve junction, calomel type.

b. Magnetic stirrer.

c. 50 ml. titration buret.

d. 100 ml. beaker

The following solutions were used:

a. Sodium o-iodosobenzoate 0.0005 N.

   132 mg. of o-iodosobenzoic acid were added to 5 ml. of 1 N NaOH and stirred until completely solubilized; then the mixture was made up to 1000 ml. with distilled water at room temperature.

b. 1 N HCl

c. 3% KI

d. 0.002 N $Na_2S_2O_3$

   0.31596 grams per liter since $Na_2S2O3 \cdot 5H_2O$ was used.

The detailed procedure was as follows:

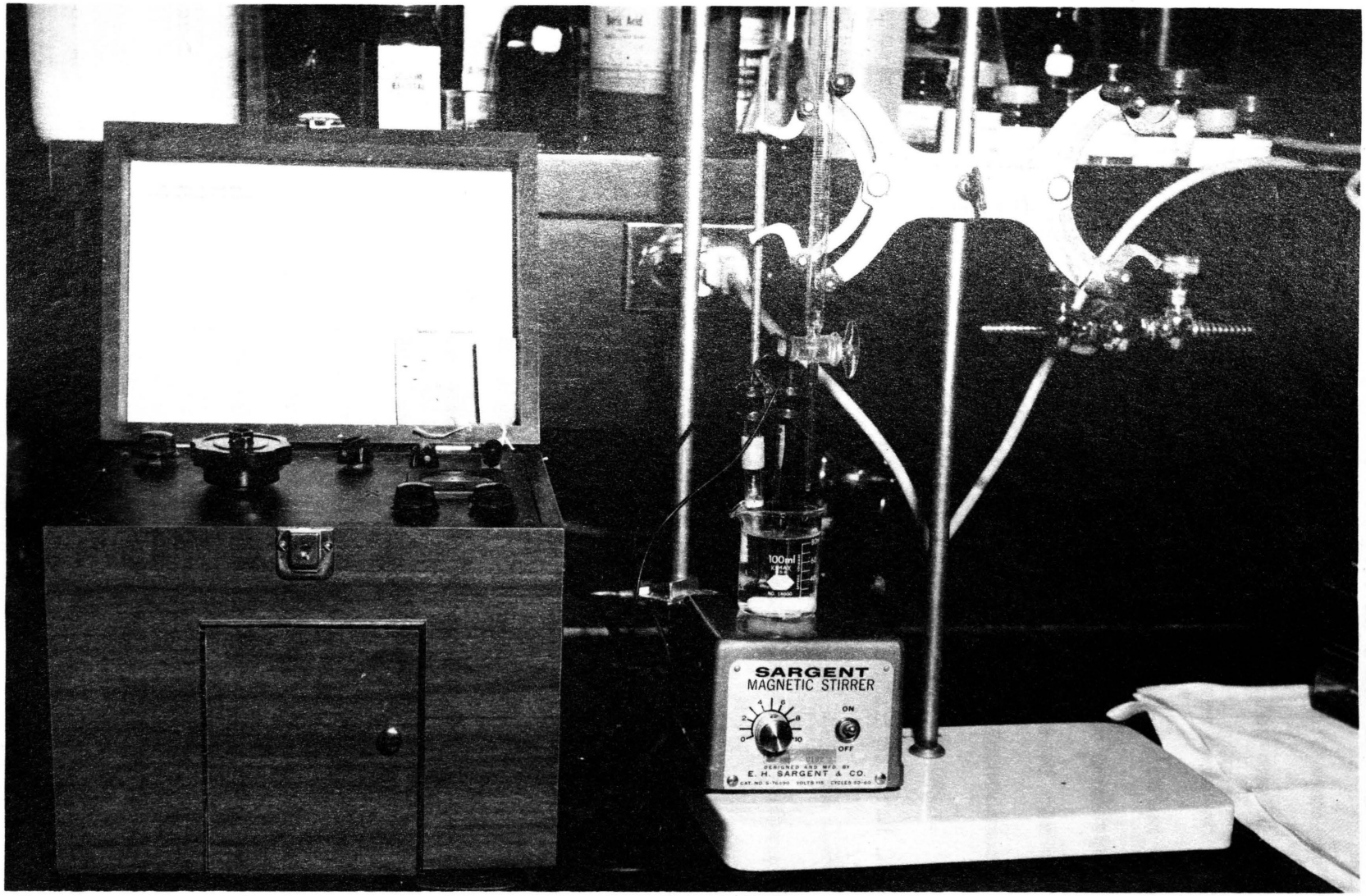


Figure 1. Apparatus used for amperometric titration of sulfhydryl groups in dairy spread by iodine and o-iodosobenzoate.

Fifteen grams of spread sample were weighed in a 250 ml. Erlenmeyer flask, approximately 100 ml. of distilled water at 45°C were poured into the flask, then this was stoppered and agitated for one minute until all the spread was solubilized. The total volume was made up to 250 ml. with distilled water at room temperature. From this solution which contained 0.06 grams of spread sample per milliliter were measured 70 ml. (4.20 g. of spread) and poured into the 200 ml. beaker of the titration equipment, followed by 15 ml. of 0.0005 N sodium o-iodosobenzoate from the buret. The mixture was gently stirred for three minutes. Meantime a flask was prepared containing 5 ml. of freshly diluted 3% KI, 8 ml. of 1 N HCl and 10 ml. of standardized freshly diluted 0.002 N $Na_2S_2O_3$. The contents of this flask then were poured and rinsed into the beaker. After 15 seconds the electrodes were immersed, the magnetic stirrer was stopped, and the millivolts of the oxidation-reduction system were measured. Resuming the agitation the mixture was titrated with more 0.0005 N sodium o-iodosobenzoate, with readings of the mV in the potentiometer taken after each addition. An average of 20 seconds was necessary after each addition to allow the reaction to be stabilized. When the needle of the potentiometer showed a constant millivoltage the reading was taken. The titration continued with more 0.0005 N sodium o-iodosobenzoate until a sharp increase in the millivoltage was observed. This point corresponded to the apparition of free iodine.

Carefully, readings were taken after the addition of every 0.1 ml. of sodium o-iodosobenzoate portion. After the free iodine liberation point, the sharp increase led to a slight constant increase of the mV.

The millivolts were plotted against the milliliters of the sodium o-iodosobenzoate. A blank was run on the solvents in exactly the same manner and this constituted a standardization of the o-iodosobenzoate against the standard thiosulfate. The subtraction of the blank milliliters from the milliliters of the titrated sample gave the amount of sodium o-iodosobenzoate consumed in the oxidation. Calculations of the cysteine percentages were made on the assumption that sulfhydryl groups were oxidized to the disulfide (44, 45).

Diacetyl Determination.

The original Prill and Hammer (81) method with some of the modifications introduced by Alfke (1) was followed in this work for the quantitative determination of diacetyl in dairy spread.

The apparatus, reagents, and procedure as used were:

a. Apparatus

The modified apparatus (1) which permitted distillation of six samples at the same time, was used here, following the recommendations of Alfke.

b. Reagents

1. Hydroxylamine acetate solution

Seventy grams of sodium acetate (Na $C_2H_3O_2 \cdot 3\ H_2O$) were dissolved in water to make 200 ml. of solution. Similarly, 35 g. of hydroxylamine hydrochloride were dissolved in water to make 800 ml. of solution. A day's supply of the reagent was prepared by mixing the two solutions in the proportion of 1 part of the first to 4 parts of the second by volume.

2. Acetone-dipotassium phosphate solution

   This was prepared by dissolving 144 g. of anhydrous dipotassium phosphate or 190 g. of its hydrate ($K_2HPO_4 \cdot 3H_2O$) in water, adding 200 ml. of pure acetone, and making up to a liter with distilled water.

3. Ammonium hydroxide

   Concentrated C.P. ammonium hydroxide (sp. gr. 0.90) was used.

4. Saturated tartrate solution

   Only amounts needed at a given time were prepared by dissolving 90 g. of potassium sodium tartrate (Rochelle salt) in 50 ml. of water by warming. This solution was kept warm (about 35°C) in order to prevent crystallization.

5. Mixture of 3 and 4

   In treating a number of samples at a time it was more convenient to add 3 and 4 together. Just before use, the mixture was prepared in the proportion of 100 ml. of the warm tartrate solution to 13.5 ml. of concentrated ammonium hydroxide. It was used immediately lest it cool and crystals separate from the solution.

6. Ferrous sulfate solution

   This reagent consisted of 5 g. of ferrous sulfate ($FeSO_4 \cdot 7H_2O$) in 100 ml. of a 1% solution of sulfuric acid. This solution was discarded whenever it showed evidence of oxidation.

7. Dilution solution

Ordinarily this solution was not required, but if dilutions were found to be necessary after the color has been developed they were not made with water but rather with a freshly prepared mixture of reagents made up in the following proportion: distilled water 63 ml.; reagent 2, 10 ml.; reagent 3, 3 ml.; reagent 4, 22 ml.; and reagent 6, 2 ml.

c. Distillation

A 20 g. sample was weighed into a 500 ml. round bottom flask. The flask was attached to the distillation apparatus with the steam inlet tube directed to one side so it was not touching the dairy spread sample. Water was running through the second condenser only since the reflux condenser was found to work better without water in the jacket, working only air as refrigerant. This gave a better equilibrium of the multiple columns. One milliliter of reagent 1 (hydroxylamine acetate) was placed in the test tube which was calibrated at 6 and 10 ml. The outlet tube of the condenser was always placed under the surface of the reagent 1 in the test tube in order to preclude losing diacetyl. A slow stream of CO_2 was admitted to prevent oxidation of acetyl-methylcarbinol to diacetyl. The CO_2 inlet valve was closed and steam admitted slowly until condensation started in the reflux column, then the inlet tube of the steam in the flask was turned down into the sample and the rate of flow of steam was regulated so that the CO_2 displaced bubbled

through the reagent 1 in the test tube at a slow rate. If too rapid rate were allowed, it would sweep diacetyl through the reagent 1 and hence cause low results. When the rate of bubbling through the test tube indicated that the CO_2 has been displaced from the flask the steam was regulated so that there was a perceptible rise of liquid in the test tube with the time required to bring the level to 6 ml. being approximately 30 minutes. Too rapid distillation flooded the water condenser. During the collection of the last 1 ml. of distillate, the test tube was lowered so that the condenser tip was slightly above the 6 ml. mark. Likewise, the rate of distillation was increased slightly during the collection of the last milliliter of distillate.

d. Development of color

As reported by Prill and Hammer (81) the tubes of distillate could be stoppered and held for a short time, until the distillation was completed and the color could be developed in the group of tubes at one time. In normal operation all the diacetyl was collected in the 5 ml. of condensate in the first test tube; however, a second 5 ml. portion of distillate was routinely collected into a second tube as a precaution against incomplete distillation of diacetyl into the first tube.

The stoppers were loosened in the tubes which contained 1 ml. reagent and 5 ml. distillate, and the tubes were heated in a water bath at 85°C for one hour. After removal from the

bath but while still warm, 1 ml. reagent 2 (acetone-dipotassium phosphate) was added to each tube. This was allowed to react 5 minutes, to fix excess of hydroxylamine, then cooled. The tubes could be held at this stage if desired.

The next step was to add 0.3 ml. reagent 3 (NH_4OH) and 2.2 ml. reagent 4 (saturated tartrate solution) or 2.5 ml. of mixed reagents 3 plus 4 (reagent 5). Next 0.2 ml. of reagent 6 (ferrous sulfate) was added and tube contents were mixed immediately by inverting. The volume was brought to 10 ml. with distilled water and the contents mixed again. Reaction was allowed for 15 minutes for full color development and stabilization. The material from the tube was transferred to a matched set of cuvettes and optical density compared with that of blank. The optical density was read on a Coleman Model 14 Spectrophotometer equipped with a purple filter (Coleman #14-214) at a wavelength of 530 mμ. The blanks were prepared by adding 5 ml. of distilled water to 1 ml. of reagent 1 in a receiving tube and developed the same as the unknown samples.

e. Standard curve for diacetyl (1)

It was not practical to use diacetyl itself as the standard because (a) absolutely pure diacetyl was not readily available, (b) it was inconvenient to weigh accurately, and (c) its solution has been reported to be unstable. Since the diacetyl in the unknown was actually converted into dimethylglyoxime in the course of the analytical procedure, there appeared to be no

objection to the use of dimethylglyoxime in place of diacetyl in making the standard solution, provided that the standard and the unknown solutions were given identical treatment in all other respects.

The standard solution was prepared by weighing 0.1349 g. of purified dimethylglyoxime (recrystallized from alcohol) and dissolving in 50 ml. of pure methyl alcohol. When all the crystals had dissolved, 30 ml. of reagent 1 (hydroxylamine acetate) were added and the volume made up to a liter with distilled water. One milliliter of the solution contained 0.1349 mg. of the dioxime, which was equivalent to 0.100 mg. of diacetyl. The dilutions needed were freshly prepared each time. The function of the hydroxylamine acetate in this solution was to repress any possible hydrolysis of the dioxime, to fix any trace of aldehyde that might possibly have been in the solvent, and to inhibit any microorganisms which might otherwise have grown in the solution.

From this solution, 0.2 ml. (0.02 mg.), 0.4 ml.(0.04 mg.), 0.6 ml.(0.06 mg.), 0.8 ml.(0.08 mg.) and 1.0 ml.(0.10 mg.), were pipetted in duplicate into test tubes graduated at 6 and 10 ml. One milliliter of reagent 1 (hydroxylamine acetate) was added to each of the tubes and the volume made up to 6 ml. with distilled water. These standards and two reagent blanks (without dimethylglyoxime) were developed for color following the instructions of the item "d" above. After the development of color for 15 minutes the contents of the tubes were trans-

ferred to the matched cuvettes and read at 530 mμ. The diacetyl content of the unknowns in parts per million (ppm) was determined by comparison to the standard curve of dimethylglyoxime according to this formula (1):

$$\frac{\text{OD (of unknown)} \times \frac{1000\ \mu g}{mg}}{\text{Slope} \times \text{weight of sample (g)}} = \frac{\mu g}{g} = \text{ppm}$$

Slope was equal to OD (optical density) over concentration of diacetyl (mg.) determined from the standard curve.

Ascorbic Acid Determination

For the quantitative determination of reduced ascorbic acid in dairy spread, Sharp's method was followed and adapted to the new kind of product, since the original procedure was for fluid milk (89). The new experimental procedure was as follows:

a. Preparation of the 2, 6-dichlorophenolindophenol solution: Two tenths gram of the highly purified 2, 6-dichlorophenolindophenol were placed in a mortar and ground to break up lumps; then 50 ml. of hot distilled water were added. Grinding was continued for one or two minutes more and the supernatant liquid decanted through a filter paper into a one liter volumetric flask. More hot distilled water was added to the residue remaining in the mortar, which was ground again and the liquid decanted into the filter. This procedure was continued until the blue colored material had passed through the filter. The solution was adjusted to room temperature and made up to the volume of one liter with distilled water.

b. Preparation of sulfuric acid solution.

Approximately 0.1 N H_2SO_4 solution was prepared by taking 285 ml. of concentrated sulfuric acid, making up to one liter, and diluting 10 ml. of this solution to one liter again.

c. Standardization of the dye solution.

Fifty milligrams of ascorbic acid were put into a 500 ml. volumetric flask and diluted to volume with distilled water. This solution was used immediately for standardization of the dye solution, since ascorbic acid is oxidized rapidly

by the catalytic action of light. To 5 ml. of the standard ascorbic acid solution in a 200 ml. beaker were added 5 ml. of the diluted sulfuric acid and then 15 ml. of water. This mixture was titrated at once with the dye solution to a light pink color which persisted for at least for 30 seconds. A blank titration was made on the acid-water mixture to the same pink color, and this figure was substracted from the sample titration value. The milligrams of ascorbic acid corresponding to one milliliter of the dye solution were obtained by dividing the amount of ascorbic acid in the 5 ml. aliquot by the number of milliliters of dye solution consumed.

d. Titration of the samples.

Twelve grams of dairy spread were weighed into a 250 ml. Erlenmeyer flask, 100 ml. of distilled water at 45°C and 25 ml. of diluted sulfuric acid were added. It was stoppered and shaken during one minute until the product was dissolved. Titration of the total amount of the solution was done at once, using a 10 ml. buret calibrated in 0.05 ml. divisions for the dye. After a small amount of the 2, 6-dichlorophenolindophenol solution was added the solution assumed a pink color. After a few seconds this pink color would fade; more dye was added then until a definite pink color remained for at least 30 seconds. This was taken as the end point of the titration. After this point had been reached the pink color remained for

several minutes.

A blank titration was performed on several diluted samples which had been allowed to stand cold several days until the ascorbic acid disappeared and a constant low value was reached. The disappearance of the ascorbic acid in the diluted sample could be accelerated by adding enough copper sulfate to make about 1 mg. per liter of Cu or by placing the solution in sunlight. Results were calculated to the basis of milligrams of ascorbic acid per 100 grams of original sample.

A few precautions were necessary. The dye solution was not stable, decreasing about 1% in strength per day; therefore it had to be restandardized at 2 to 4 day intervals and daily values for its strength obtained by interpolation. Solutions over two weeks old did not give sharp end points.

The ascorbic acid solution also was not stable under ordinary conditions, so it was used within one to five minutes after its preparation. The addition of acid to the sample accelerated the destruction of ascorbic acid, so this was another reason why the titration had to be done as soon as possible.

Protein Content

The Kjeldahl method for nitrogen was used in this modified form:

a. Filter papers, S & S No. 604, were rinsed in paraffin wax and

weighed accurately on an electrical single pan Mettler balance.

b. About 2 g. of sample were weighed on the filter paper.

c. The digestion mixture contained 10 g. potassium sulfate and 0.3 g. copper sulfate.

d. The digestion mixture, sample, and 30 ml. H_2SO_4 were put in an 800 ml. Kjeldahl flask.

e. The flasks were put on a Laboratory Construction Company electrical variable heat Kjeldahl digestion unit.

f. The flasks were rotated every 10 minutes until the mixture was digested off the sides of the flask.

g. The digestion was continued for half an hour after contents appeared "green".

h. The flasks were cooled, and 250 ml. distilled water was added, after which the flasks were cooled again.

i. Erlenmeyer receiving flasks with 50 ml. 4% boric acid solution were prepared.

j. Sodium hydroxide solution (40%) was added (80-85 ml) to the sample in the flask. Three pieces of zinc shot were added carefully. The flask was connected to a distillation apparatus and delivery tubes were placed in the receiving flasks containing the boric acid solution. Then the heating elements were turned on.

k. Approximately 250 ml. of distillate were collected.

l. Three drops of 0.1% methyl red-methylene blue indicator in alcohol were added, and the distillate titrated with 0.0715 N sulfuric acid until a steel gray color was obtained.

m. A blank containing digestion mixture and acid plus one gram of sugar was given the same treatment.

The formula used for the calculation of nitrogen % was as follows:

$$\frac{\text{ml } H_2SO_4\text{(sample)} - \text{ml } H_2SO_4\text{(blank)} \times \text{Normality acid} \times 0.014 \times 100}{\text{Sample weight}} = N\%$$

% protein = % N x 6.38.

Starch Gel Vertical Electrophoresis.

In this research work was used a Starch Gel Vertical Electrophoresis Apparatus designed by Samuel H. Boyer (10), The John Hopkins Hospital and University, and prepared by Buchler Instruments, Inc. This apparatus differed from that described by Smithies (91) in that the samples were inserted from the under side of the gel mold into depressions made by a comb held in a removable plastic cover (11). Insertion from the underside of the gel permitted the use of a plate glass for covering the molten gel which assured the formation of two co-planar surfaces and consequently provided a uniform cross section. The depth of the sample slots was fixed by the removable comb at the time of pouring the gel which eliminated the possibility of gel perforation by slot formers. The gel remained within the mold from its preparation until the electrophoretic run was finished so distortions produced by gel handling during the insertion of the sample were avoided.

The materials used were:

1. Vertical gel mold, with plate glass cover for receiving the molten gel
2. Two electrode vessels

3. Flannelette for use as wicks.
4. One lucite stand.
5. Small volume pipets.
6. One regulated power supply unit for constant voltage/constant current operation regulated to a maximum of 1000 VDC and 200 mA for use on 115VAC, 60 cps (Buchler Instruments Inc. 3-1014 A).
7. Gel slicing device.
8. Staining boxes.
9. Potato starch. This may be hydrolyzed by acid-acetone as described by Smithies (91) or already hydrolyzed. Connaught Starch Hydrolyzed supplied by Fisher Scientific Co. under catalog No. S-576 was used in this work.
10. Stain - Amido black (Buffalo black).

The preparation of vertical starch gels was according to Boyer's instructions (10). Sixty-one grams of hydrolyzed starch were put into 200 ml of Tris-Citrate buffer solution contained in 1000 ml wide mouth Pyrex filtration flask. The Tris-Citrate buffer solution was previously prepared by dissolving 9.2 grams of Tris (hydroxymethyl) amino-methane and 1.05 grams of citric acid in one liter of distilled water and adjusting the final pH to 8.6 with about 50% NaOH solution. The flask was constantly swirled during the addition of the starch to the buffer solution. The lip and walls of the flask were washed with additional 300 ml of buffer solution. This way of preparation of the starch suspension avoided "lumpiness" in the resulting gel. The filtration flask containing the starch-buffer suspension was then

vigorously heated over a gas flame and the contents were constantly kept swirling so as to avoid burning the starch and to exclude lumpiness. After four to five minutes the flask contents became quite viscous and then, after several minutes of continued heating and swirling, began to clear. Heating was continued until the first bubbles of boiling began to form. The flask was plugged with a solid neoprene stopper and at once degassed by applying suction to the side neck tubulation. Degassing was continued until vigorous boiling with rapid formation of large bubbles developed and persisted for 15-30 seconds. On breaking the vacuum a bubble free transparent gel resulted. The stopper was removed and the gel at once was poured into the Lucite mold starting at the end away from the slot formers and allowing the gel to flow toward the other end and slop over along the sides and at the end adjacent to the slot former. A previously warmed one-sixteen inch thick glass plate (hot to the touch) was promptly applied to cover the gel. Then at room temperature the gel was allowed to cool for two to three hours in the absence of air drafts. When the completed gel was opaque and cool, it was inverted carefully, with the end of the mold bearing the higher end piece projecting over the edge of a table top. The gel slopover along the edges was gently removed with a spatula. The screws holding the end pieces were loosened but not removed and then, just before the sample insertion, the comb bearing the slot formers was gently removed.

The samples were prepared to contain about 50 mg. of dry matter from the spread per milliliter. Samples were inserted with a Pasteur pipet with narrow tip. With special care to remove any bubble, the

tip was introduced into the slot formed in the gel with a single sliding motion and held at 45° angle. The slots were filled to a point approximately 0.5 mm below the surface of gel exposed by the removal of the slot former. Approximately 0.040 to 0.045 ml. were required to fill each slot. After all slots were filled, with care to avoid spillage from one slot to another, they were covered with a new freshly prepared gel with exactly the same concentration. In a few minutes the covering gel was solidified. In this way the liquid samples remained trapped within the gel. When this sample covering was cooled and hardened the end pieces of the mold were carefully removed and the mold was stood upright in the lower wick chamber. Connection to the upper wick chamber was made by a piece of flannelette wetted with the chamber buffer. In the research work several pH's and buffer solutions were tested. The best results were obtained with the same Tris-Citrate buffer at pH 8.6 used for the preparation of the starch gel and with 7 M urea buffer of pH 8.6. Special care was taken to ensure that the gel was precisely perpendicular in order to avoid distortion of the electrophoretic display. When they were exactly placed, the gel and mold was tightly locked in place with a metallic spring retention. Then the electrophoresis run was started at room temperature using a low amperage DC source with a constant voltage during the operation. Running times of 14-16 hours were sufficient for the separation of the milk proteins.

On completion of electrophoresis the glass covering plate was gently removed. Using a spatula blade the terminal two inches of the gel from both ends were sliced and discarded. Supported by the hand,

the gel was then gently lifted from the mold and its under surface briefly blotted by placing on a paper towel. The gel was then transferred without inversion to the slicing surface. The purpose of the slicing was to permit examination of the interior surface. Boyer (10) stated, "Electrophoresis on the surface of starch gel is much obscured by distortions produced in part by the more rapid migration of materials through a thin layer of surface fluid and in part produced by alterations in electrical flux occuring at boundaries. The result is considerable smearing of the electrophoretic display. Examination of the interior surface of a longitudinal gel slice largely avoids such distortions." The gel was sliced in two sections using a Buchler Gel Slicer with an 0.008-inch piano wire. The optimum thickness was 2.5 to 3 mm from any exterior surface (the total thickness was about 7-9 mm) yet still within the range of sample slot depth. The two halves of the gel were carefully separated with a spatula and put, cut surfaces uppermost, into a staining box. The staining solution used was naphthalene black (syn., naphthol blue black, Buffalo Black, Amido-Schwarz stain), 400 mg in one liter of solution composed of 5 volumes of methyl alcohol, 5 volumes of distilled water and one volume of glacial acetic acid. The gel was stained by covering its surface with the naphthalene black solution during two minutes, then the gel was washed with acid methanol water solution and allowed to develop fully by remaining in the washing solution overnight in a covered container.

Polyacrylamide Vertical Electrophoresis (74).

An attachment to the Buchler vertical starch gel electrophoresis

apparatus was designed at the National Institutes of Health, Bethesda, Maryland and was constructed to adapt the apparatus for use with large acrylamide gel columns. The same stand, wick, and electrode chambers of the apparatus were used. The gel mold was removed and in its place an acrylic plastic attachment for eleven tubes was hooked to the stand. Eleven (or the desired number of) acrylic plastic tubes containing the gel columns prepared as described below were fixed in position on the attachment.

Gels were prepared according to Ornstein and Davis directions (72):

a. Solutions

Solution 1

75 g. Acrylamide.

2 g. N-N-Methylenebisacrylamide.

0.038 g. Potassium ferricyanide made up to 250 ml. with water.

Solution 2

24 ml. 2 N HCl.

36 g. 2-Amino-2-hydroxymethyl-1, 3 propanediol (Tris buffer).

0.46 ml. N,N,N',N',-Tetramethylethylenediamide made up to 250 ml. with water

Solutions 1 and 2 separately were stable for months in the refrigerator. However, solutions before use were examined for precipitates which eventually formed.

Solution 3

Mix equal parts of solutions 1 and 2; if refrigerated,

this was stable for several weeks.

Solution 4

As catalyst for gelification of the solution 3, a 0.14% ammonium persulfate solution was prepared. For eleven columns approximately 80 ml. were necessary.

Solution 5

20 g. Acrylamide.

5.0 g. N-N-Methylenebisacrylamide.

1.71 ml. 86% Phosphoric acid.

5.7 g. 2-Amino-2-hydroxymethyl-1-1, 3-propanediol (Tris buffer) - 0.004 g. riboflavin made up to 400 ml. with water.

Note: Since riboflavin served as a photocatalyst for polymerization, the other ingredients were first combined in 300 ml. of water. Riboflavin was taken up in 50 ml. water and mixed into the previously prepared solution which was then made up to 400 ml. with water. Solution was kept in dark bottles. If refrigerated, it was stable for several weeks. For use, it was diluted with equal volumes of water.

Solution 6

Buffer for compartments. Stock solution.

30.28 g. Boric acid

37.30 g. Sodium tetraborate dissolved in 2000 ml. water.

For use it was dissolved in equal volume of water.

The buffer stock solution in higher concentrations and stored in the cold has the tendency to crystallize.

b. Preparation of the columns

The bottoms of the eleven (or less) clean and dry tubes were stoppered with the specially-shaped, silicone rubber stoppers. By use of the upper stopper and the holding spring on the electrophoresis stand, they were fixed in exact vertical position on the stand.

Seventy five milliliters of solution No. 3 with 75 ml. of the persulfate solution No. 4 were mixed in a 250 ml. aspirator flask. When the solutions were cold, they were warmed up to 25-30°C, and the air removed by evacuation.

By use of separatory funnels the solution was then added into the tubes until the lowest mark was reached. It was important that the presence of air bubbles be avoided. Special care was taken in filling all the tubes to equal levels in order to obtain equal mobilities. The solution was then overlayed with a few milliliters of distilled water by using a Pasteur pipet with the tip folded at 90° angle. By dipping this end into the acrylamide liquid and releasing a horizontal water flow by gentle opening of the finger, a sharp interphase could be created. The tubes were left at room temperature for 30 minutes to one hour for polymerization of the acrylamide. This process, completed below the water-gel interface, yielded a completely horizontal surface of the polymerized material. Slight warming of the columns was experienced during the poly-

merization. The water on the top of the column was eliminated by reversing the tubes and blotting the droplets on the outside with paper. Five milliliters of solution No. 5 were mixed with 5 ml. of water and the tubes above the gel were rinsed with this solution. The tubes were then placed back on the electrophoresis stand as before. Thirteen milliliters of solution No. 5 were measured in a cylinder and 13 ml. of water were added. The mixture was poured into an aspirator flask and air was removed by evacuation. When photopolymerization did not progress with sufficient ease, a drop each of solutions No. 3 and No. 4 were mixed into the 26 ml. mixture. From this mixed solution, 2 ml. were pipetted into each tube and overlayed with a small amount of water. Then the liquids were exposed to a strong fluorescent lamp at a distance of a few inches.

Turbidity and a sharp line several millimeters under the meniscus indicated the completeness of gelification in approximately 30 minutes. Excess water was removed by careful inversion of the tubes, in order not to displace or break the gel, which might allow air between the wall and gel. Any remaining liquid around the orifice of the tube was blotted with paper.

Sample solutions were prepared to contain approximately 2 to 10 mg. of dry matter per milliliter.

When light polymerization was obtained by proceeding as described above, one milliliter of sample solution was pipetted into

a test tube containing 1.1 ml. of solution No. 5; then one drop each of solutions No. 3 and No. 4 were added. Then 2 ml. of this mixture were pipetted onto the top of the previously prepared gel. All this was again overlaid with a few droplets of water and exposed to light for polymerization.

c. The electrophoretic run

When polymerization of the sample was completed, a sharp line was formed below the meniscus. The excess liquid was again separated as described above.

The two buffer compartments were placed on the stand and the tube holding attachment was brought into position by the use of two hooks. The lower chamber was filled with approximately 400 ml. of diluted, pre-cooled buffer; and the upper chamber with approximately 800 ml. of the same solution.

The silicone rubber stoppers were removed from the bottom of the columns. The columns were then tilted and dipped into the buffer of the upper compartment in order to fill with buffer solution the space left by removal of the stoppers and eliminate air. The tubes were then inserted into the holes of the attachment and fixed in position by the stoppers on the upper end. When all tubes were in place (if a smaller number of tubes was used, the appropriate numbers of stoppers were placed in the empty holes) buffer was added to approximately the same level as in the upper buffer compartment. With the flannel wick soaked in buffer, an electric bridge was formed. The protective cover was then

placed over the equipment and the whole assembly was ready to start the electrophoretic run. Power supply was set to constant voltage and electrophoresis was allowed to proceed for twelve to sixteen hours. Figure 2 shows an electrophoretic run in progress.

d. Processing of the acrylamide gel columns after electrophoresis. After termination of the run, buffer from the attachment was drained by removing the outlet tube plug. Columns were removed by gentle water pressure with the special instrument also fabricated by Buchler (#3-1077) and shown in Figure 3. The acrylic plastic material of the tubes and the specially-shaped end of the columns formed by the silicone rubber stoppers made the removal of the gel columns fast and safe from damage. Columns were placed on a tray, and after excess liquid was drained off they were marked with black India ink on their anodic end, outside of the electrophoretic pattern. Fixation was performed in a solution of 47% methyl alcohol, 47% water, and 6% acetic acid for several hours. After the fixation of the proteins, they were visible when observed against a black colored surface. However, for greater clarity, the columns were stained. The staining solution was prepared with 400 mg. amido black (Buffalo black) per liter in a 5% acetic acid solution. The best staining time was determined to be 8 minutes. Finally, the destaining procedure, which left the colored proteins only, was done by allowing the gels to remain overnight in a 2% glacial acetic acid solution.

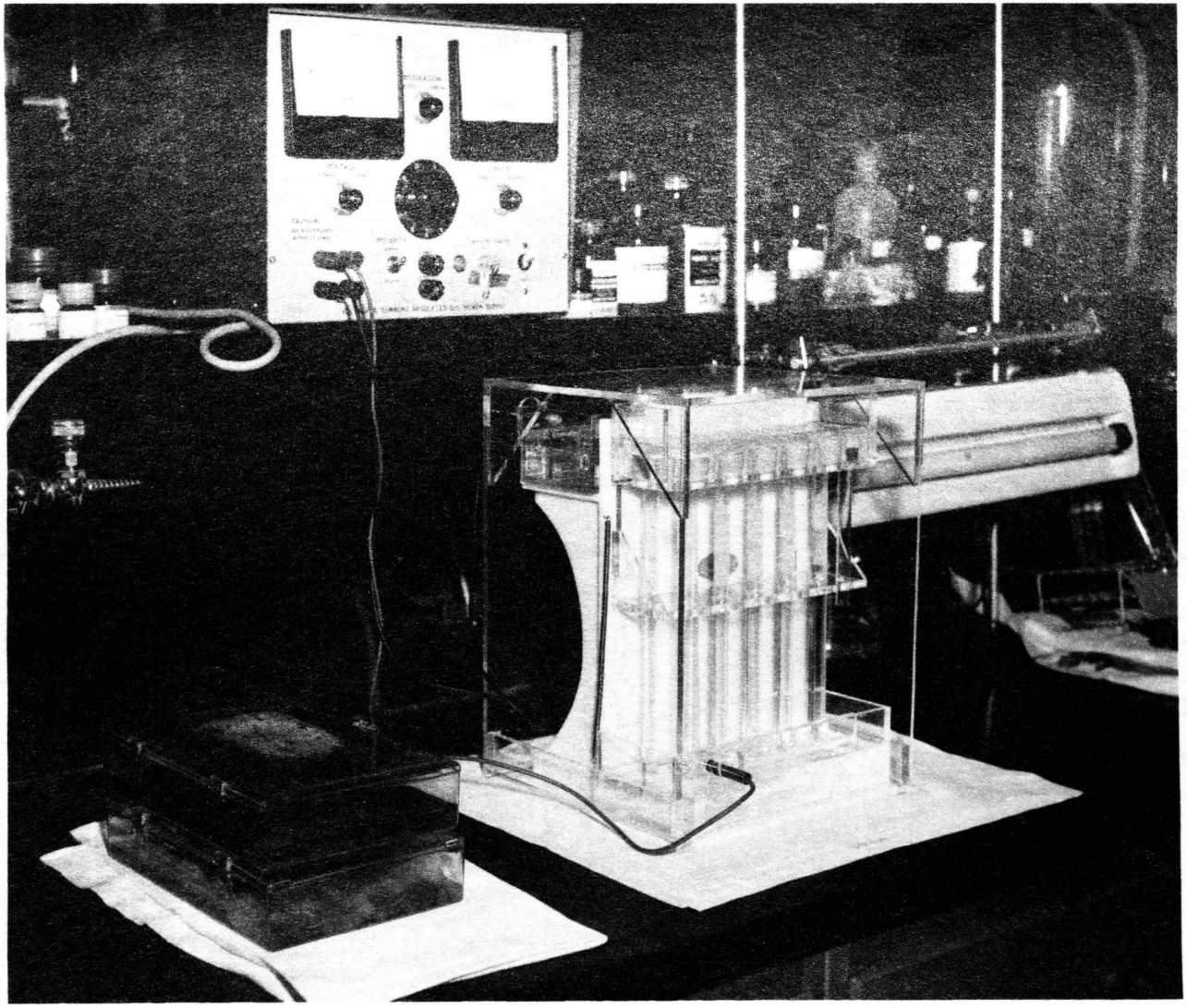


Figure 2. Polyacrylamide vertical gel columns electrophoresis apparatus running samples of dairy spread.

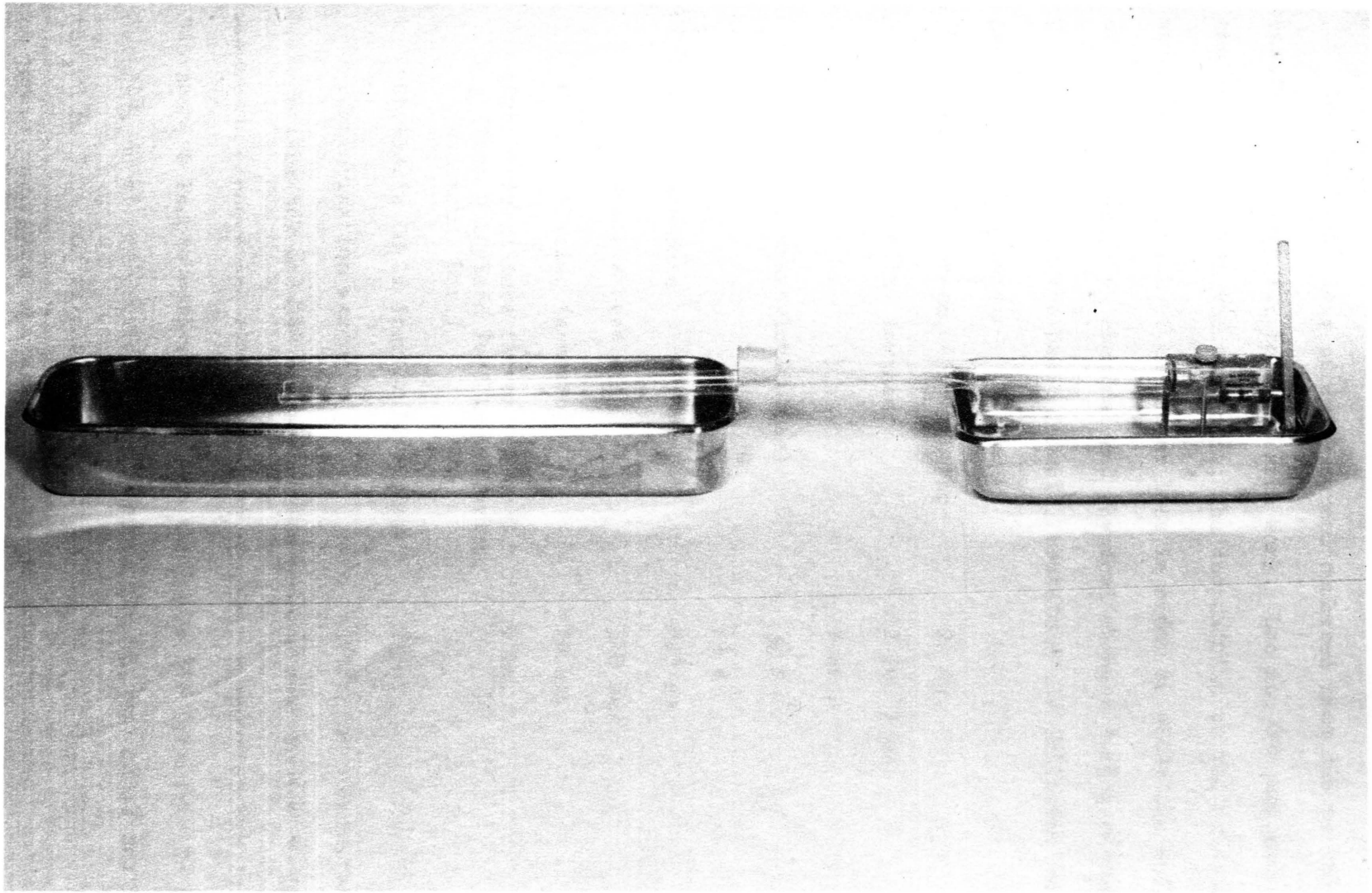


Figure 3. Water pump extractor for gel columns in polyacrylamide vertical electrophoresis.

Preparation of Dairy Spread

Ten different lots of dairy spread numbered from 346 to 355 were specially prepared for this research work. Lot no. 346 was made by the regular formula and procedure; the composition or the processing were changed in each of the other lots in order to study the influence of various factors on chemical and physical changes during storage.

Lot 346, with the basic formula, contained the following ingredients and amounts:

Pasteurized cream, 45% milk fat	27 lb.
Nonfat dry milk, low heat	2 lb. 7 oz.
Salt	6 oz.
CMC (Carboxymethylcellulose)	13 g.
Gelatin	13 g.
Sodium benzoate	8.5 g.
10% β-Carotene beadlets	350 mg.
Butter coloring (annatto)	20 ml.
Synthetic culture flavor DCF-85 B (from Dairyland Food Laboratories, Waukesha, Wis.)	4 ml.
Chumlea's CH-11 Starter Distillate	21 ml.
Pasteurization was at	165°F for 30 minutes

In lots 347 and 348 pasteurization process was changed to 175°F during 30 minutes and 178°F during 40 minutes, respectively. In lots 349 and 350 lactic acid was added to the basic 346 formula. To the lot 349, 40 ml. of lactic acid diluted to 100 ml. was added and lowered the pH to 5.3; and to the lot 350, 25 ml. of lactic acid also diluted to 100 ml. was added instead, giving a pH of 5.6.

In lot 351 the nonfat dry milk as source of nonfat solids was omitted, instead 7 lb. of condensed skimmilk was used. The fat was from two sources: pasteurized 45% cream, 14 lb. 11 oz., and butter 7 3/4 lb. Calculations were made in order to maintain the same fat and solids-not-fat levels. The butter coloring in this lot was reduced to 10 ml.

In lot 352, the fat source was 24 lb. unpasteurized 43% cream plus 2½ lb. of butter. Also 220 ml. of water was added to assure the same total weight of the lot.

In lot 353, 15 g. of ascorbic acid was added to the same formula as for lot 352. The acid was diluted in 220 ml. of water before being added to the other ingredients. The final pH of the lot was pH 5.75.

In lot 354 the NFDM was changed from low-heat to high-heat, the same amount: 2 lb. 7 oz.

In lot 355 the composition was exactly the same as lot 346. The pasteurization also was performed at 165°F during 30 minutes but in this case with recirculation of the batch. For this purpose a centrifugal pump was used which moved the mix constantly taking from the bottom and refeeding the product back into the vat. Exposure to the air promoted oxidation, and mechanical friction was afforded by the pump and pipelines.

RESULTS AND DISCUSSION

Protein Sulfhydryl Groups.

The cysteine content of the samples was determined by using the Beckman 2000 Model G pH meter and titration with sodium o-iodosobenzoate. As mentioned in the review of literature, cysteine is the principal site of the sulfhydryl groups in proteins. Because of the relatively small amount of cysteine in the protein content, which itself was only about 5% in the dairy spread, it was necessary to determine by experimentation the concentration of sodium o-iodosobenzoate which was suitable for the titration. After several trials the most suitable normality of the sodium o-iodosobenzoate was determined to be 0.0005 N.

Starting from the readily available o-iodosobenzoic acid ($C_7H_5IO_3$), 0.0005 N sodium o-iodosobenzoate was prepared according to the procedure on page 38 . One milliliter of this last solution contained, therefore 0.132 mg. since the molecular weight of the acid is 264.03. Samples were prepared containing 0.06 g. dairy spread per milliliter and the volume used in the titration was 70 ml. (4.20 grams of dairy spread). Calculations of cysteine percentages and parts per million were made on the assumption that sulfhydryl groups of cysteine were oxidized to disulfide cystine (44, 45) according to the reaction on page 18.

Example of calculation:

Two moles of cysteine reacts with one mole of sodium o-iodosobenzoate.

Cysteine, eq. wt. 121 1 meq. = 0.121 grams.

Amount of sample titrated: 4.2 grams.

Titrating reagent : Sodium o-iodosobenzoate 0.0005 N

Milliequivalents of titrating reagent:
ml. reagent x 0.0005 = no. meq.

Actual wt. of cysteine in sample titrated:
no. meq. x 2 moles x 0.121 = g. cysteine in sample

Mg. cysteine per gram of sample =

$$\frac{\text{g. cysteine in sample} \times 1000}{4.2}$$

Mg. cysteine per 100 g. of sample = mg./g x 100

Cysteine p.p.m. = mg./g. x 1000

All the samples were titrated in triplicate. After the addition of each 0.1 ml. of sodium o-iodosobenzoate the millivolts were read. Average values are shown in the Appendix in Table 1 A to 11 A.

Titration of a blank solution used 19.6 ml. of sodium o-iodosobenzoate, which corresponded to the point of liberation of free iodine. This point was easily observed also in the blank solution by its characteristic yellow color, because it was not masked. The needle of the potentiometer showed its maximum deflection exactly when the yellow color just appeared and corresponded to the inflection points of the curves when the millivolts were plotted against the milliliters of 0.0005 N sodium o-iodosobenzoate consumed in the iodine liberation. Examples of the resulting graphs are shown in Figure 4 and Figure 5 which corresponded to the blank titration and to a typical example of dairy spread titration (lot 349), respectively.

The reducing capacity of the ten dairy spread lots calculated as explained above, are shown in Table 1.

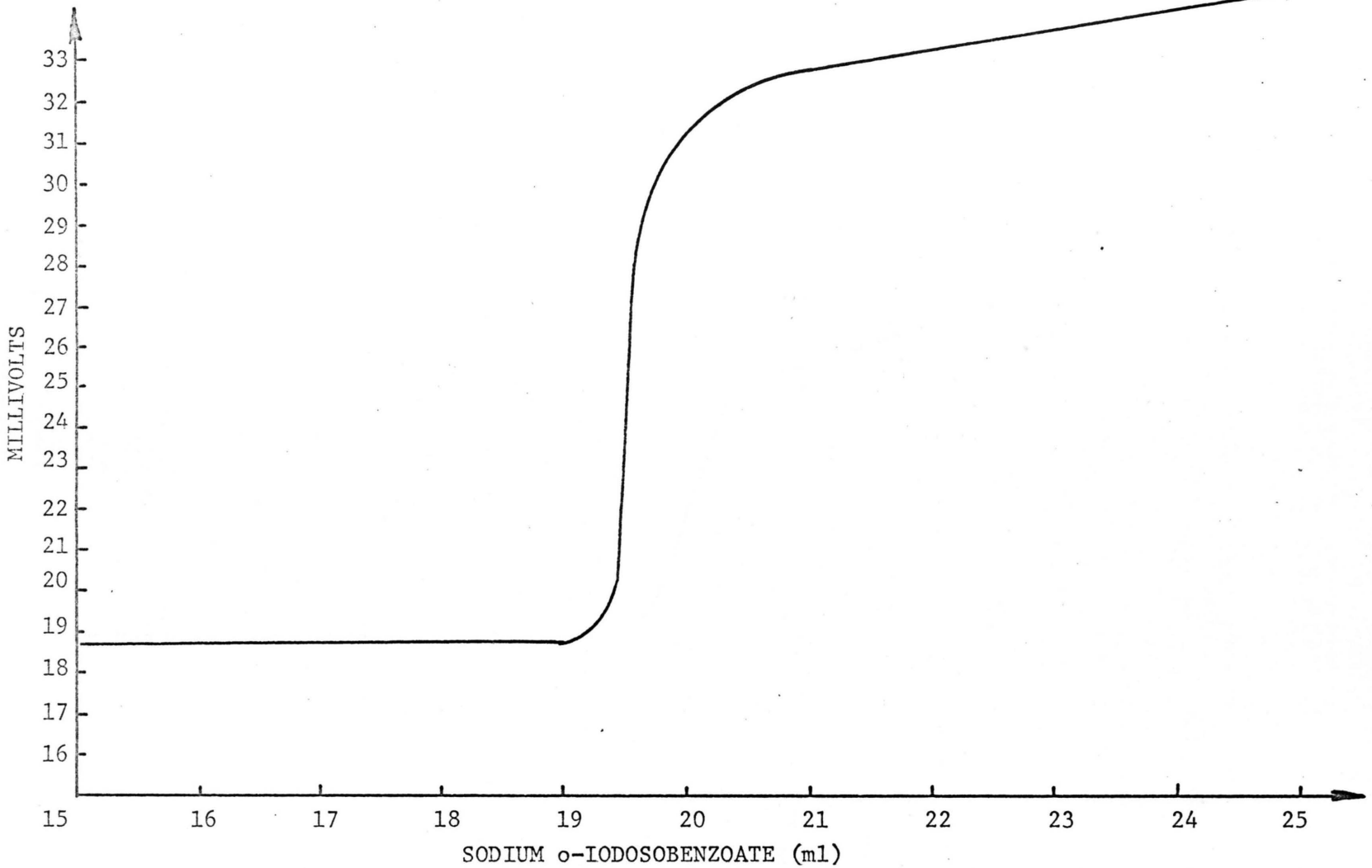


Figure 4. Relationship of 0.0005 N sodium o-iodosobenzoate addition to electrical potential during titration of reagents for -SH determination in dairy spread.

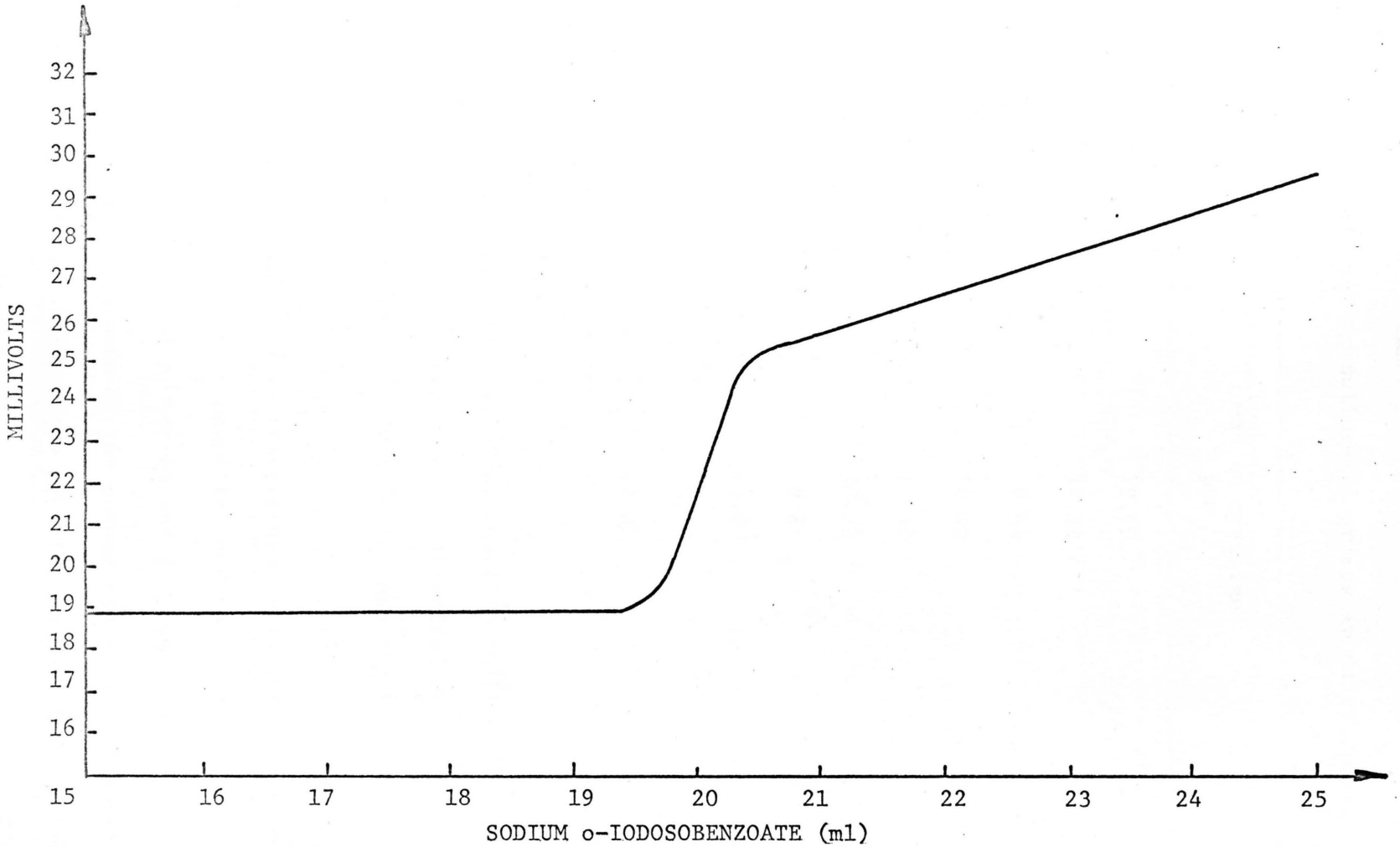


Figure 5. Relationship of 0.0005 N sodium o-iodosobenzoate addition to electrical potential during titration of -SH groups in dairy spread lot 349.

TABLE 1

Reducing capacity of protein sulfhydryl groups in dairy spread

Lot No.	-SH as cysteine ppm
346	79.90
347	17.28
348	8.64
349	14.40
350	14.40
351	17.28
352	2.88
353	28.80
354	0.00
355	31.39

Lot 346 showed the highest content of reactive sulfhydryl groups. This lot had the basic composition and was pasteurized at 165°F for 30 minutes. Low heat NFDM was used in this lot and the heat treatment for this kind of milk powder is generally not less than 71°C (161°F) for at least 15 seconds (39). This temperature falls into the range where the protein structures start uncoiling and the sulfhydryl groups have more reactivity, as stated by Larsen and Jenness (59).

The pasteurization treatment of the spread mix at 73°C (165°F) for 30 minutes apparently continued the protein denaturation and the

subsequent uncoiling of its helix structure increased the -SH reactivity.

A substantial decrease of the sulfhydryl groups reactivity was observed in lots 347 and 348. The higher temperature treatment in the pasteurization process of these lots (175°F for 30 min. and 178°F for 40 min., respectively) probably was the cause of lower values. This would confirm the work done by Larsen, Jenness and Geddes (56) on the effect of heat treatment on the sulfhydryl groups of milk serum proteins, and Zweig and Block (109) on the effect of heat treatment on the sulfhydryl groups in skimmilk and nonfat dry milk. The results indicated that the loss of sulfhydryl groups was a function of time and temperature, when the latter was above the "critical temperature". According to Zweig and Block (109), the critical temperature coincided with the appearance of cooked flavor. Above that temperature the sulfhydryl concentration decreased very rapidly with increasing temperatures.

In lots 349 and 350 the addition of lactic acid produced a similar decrease of the sulfhydryl groups' reactivity. The pH's of these lots were 5.3 and 5.6 respectively, which was quite more acidic than was the standard lot 346 with pH 6.05.

In lot 351 with condensed milk as source of milk solids the same result was obtained in cysteine content as lot 347, which was pasteurized at 175°F for 30 minutes. The heat treatment of the added condensed skimmilk probably was a little higher than that of NFDM, hence the sulfhydryl reducing capacity was lowered.

Lot 352 showed very low reducing power against sodium o-iodosobenzoate. In this lot unpasteurized cream and butter were used as

fat sources, but no apparent relation to this low cysteine content could be attributed to the butter and cream. To lot 353, which had the same mix formulation as lot 352, ascorbic acid (15 grams) was incorporated, which lowered the pH of the mix to pH 5.7. The lower pH or the presence of the ascorbic acid, modifying the redox potential, had definite influence on the sulfhydryl reducing capacity, for it was higher than that of lots 347 to 352.

Lot 354 was prepared with high heat NFDM powder. No reducing capacity was found in titrating the cysteine content. Presumably the reason was the excess of temperature in the heat treatment process of the high heat nonfat milk powder which likely volatilized the sulfhydryl groups as H_2S.

The last lot, number 355, with the same composition as the standard 346 and the same pasteurization process, had less than half of the reducing power of the standard. This was attributed to the recirculation and air oxidation to which the lot was submitted. Larson and Jenness (58) reported that probably the decrease of reducing capacity following treatment at higher temperatures is due to oxidation, since it was largely prevented by excluding air from the sample during heating. Lot 355, exposed to higher oxidation by intense recirculation during the heat treatment, confirmed Larson and Jenness' results, since the cysteine parts per million were reduced by 61% compared with cysteine content of a similar lot (346) which had natural but not provoked air oxidation.

Oxidation-Reduction Potential

Changes in oxidation-reduction potential of the ten lots of dairy spread were measured every 30 days during three months. The samples were stored in the Dairy Products Laboratory refrigerated chamber at 40°F following their fabrication. Before readings were taken, the samples were exposed several hours at room temperature in their closed containers, so that the oxidation-reduction potential was read at 25°C.

A Beckman Model G pH meter was used with a platinum electrode and a sleeve type calomel reference electrode. In accordance with Beckman Instruments instructions, only the lower part of the glass sleeve was immersed in the samples. The results are expressed in Table 2.

Lot 346, which had the standard formula and pasteurization, had an almost constant level of oxidation-reduction potential during 60 days storage, although it increased in the third month. This constant oxidation-reduction potential reflected a fairly good keeping quality during two months, but the tendency for the potential to increase after that period indicated that marked physico-chemical changes were occurring.

The heat treatment apparently reduced slightly the oxidation-reduction potential in lots 347 and 348 which were treated at higher temperatures (175°F and 178°F, respectively). That this phenomenon also happens in milk was reported by Eilers and Saal (30), Harland and Coulter (41) and Neurath, Greenstein, Putnam and Erickson (71) who stated that the lowering of the oxidation-reduction potential

TABLE 2

Oxidation-reduction potential of dairy spread when freshly prepared and after storage at 38°F for 30, 60 and 90 days

Lot no.	Fresh		30 days		60 days		90 days	
	E*	E_h*	E	E_h	E	E_h	E	E_h
346	54.5	298.8	56.0	300.3	55.7	300.0	59.0	303.3
347	49.3	293.6	58.2	302.5	58.0	302.3	58.2	302.5
348	51.1	295.4	55.4	299.7	58.4	302.7	58.2	302.5
349	54.8	299.1	52.4	296.7	58.5	302.8	58.9	303.2
350	54.0	298.3	55.2	299.5	58.1	302.4	59.4	303.7
351	52.8	297.1	52.0	296.3	58.4	302.7	58.0	302.3
352	52.2	296.5	51.4	295.7	56.3	300.6	56.3	300.6
353	42.0	286.3	38.4	282.7	42.8	287.1	35.1	279.4
354	50.1	294.4	52.4	296.7	50.8	295.1	45.5	289.8
355	57.2	301.5	44.2	288.5	54.4	298.7	51.1	295.4

* E was the measured voltage in mV.

E_h was the oxidation-reduction potential in +mV.

coincided with the liberation of sulfhydryl groups. Hence, when figures in Table 1 and Table 2 of this paper were compared, it appeared that the liberation of sulfhydryl groups, which decreased the cysteine and cysteine-aggregates content, occurred in lots 347 and 348.

The addition of lactic acid to lots 349 and 350 and condensed skimmilk and butter to lot 351 did not affect the oxidation-reduction

appreciably in the fresh product potential compared with lot 346. These lots showed a gradual high increase in their O-R values after 60 days similar to those of lot 346.

In lot 352 in which unpasteurized cream was used the oxidation-reduction potential was somewhat lower initially. Again there was a marked increase in the readings at 60 days, but at the last readings the potential was slightly lower than the values for the above lots.

Ascorbic acid gave a markedly lower oxidation-reduction potential in lot 353, as was expected from the basic equation discussed in page 10.

$$Eh = Eo + 0.06 \log \frac{[Ox]}{[Red]}$$

Ascorbic acid is the reduced form whose oxidation yields dehydroascorbic acid, consequently the $\frac{[Ox]}{[Red]}$ ratio was lowered by ascorbic acid addition and so was the oxidation-reduction potential E. Upon storage, lot 353 displayed an even lower oxidation-reduction. The reason might have been the irreversible transformation of the oxidized form, dehydroascorbic acid, to diketogulonic acid, which takes place upon hydrolysis and occurs always except in rather acid media. The concentration ratios therefore were decreased during storage time since the oxidized form was disappearing.

Lot 354, with high heat NFDM, when freshly produced yielded a slightly lower oxidation-reduction potential and this coincided with the total liberation of sulfhydryl groups which one would have expected in a highly heated product. It appeared that heat treatment promoted these two linked behaviors, which confirmed previous works on milk (30, 41, 71).

Finally, in lot 355, which was exposed to mechanical agitation and air oxidation, the highest oxidation-reduction potential of all the batches was observed in the fresh product. This was reasonable since it was logical to suppose that the majority of the ascorbic acid present was oxidized to dehydroascorbic, which increased the ratio of oxidized to reduced forms and consequently the oxidation-reduction potential. Storage again, as in lots 353 and 354, favored the irreversible hydrolysis of dehydroascorbic acid and decreased slightly the ratio and the oxidation-reduction potential according to the above formula.

Diacetyl Determination

Diacetyl, which was in dairy spread through the addition of starter distillate plus a synthetic flavoring material, was determined following the Prill and Hammer (81) method as modified by Alfke (1).

The standard curve for diacetyl was developed first according to the instructions under item "e" of diacetyl experimental procedure on page 45. Each milliliter of the standard solution prepared with dimethyl-glyoxime was equivalent to 0.1 mg of diacetyl.

Table 3 indicates the order of reagents followed, in duplicate sets of six test tubes each one, and the optical densities at 350 mμ read in a Coleman Model 14 Spectrophotometer.

Figure 6 shows the diacetyl standard curve in which optical densities were plotted against concentrations. The slope of this curve was 6.00.

TABLE 3

Reagents used and optical densities obtained
with known amounts of diacetyl by a modified Prill and Hammer procedure.

Test tube no.	1 (blank)	2	3	4	5	6
Diacetyl,mg	0	0.02	0.04	0.06	0.08	0.10
Standard solution, ml	0	0.20	0.40	0.60	0.80	1.00
Distilled water,ml	5.00	4.80	4.60	4.40	4.20	4.00
Reagent 1, ml	1.00	1.00	1.00	1.00	1.00	1.00
Reagent 2, ml	1.00	1.00	1.00	1.00	1.00	1.00
Reagent 3, ml	0.30	0.30	0.30	0.30	0.30	0.30
Reagent 4, ml	2.20	2.20	2.20	2.20	2.20	2.20
Reagent 6, ml	0.20	0.20	0.20	0.20	0.20	0.20
Distilled water,ml	0.30	0.30	0.30	0.30	0.30	0.30
Total volume, ml	10.00	10.00	10.00	10.00	10.00	10.00
Optical density,set 1	0.000	0.067	0.210	0.368	0.480	0.570
Optical density,set 2	0.000	0.160	0.280	0.470	0.495	0.640

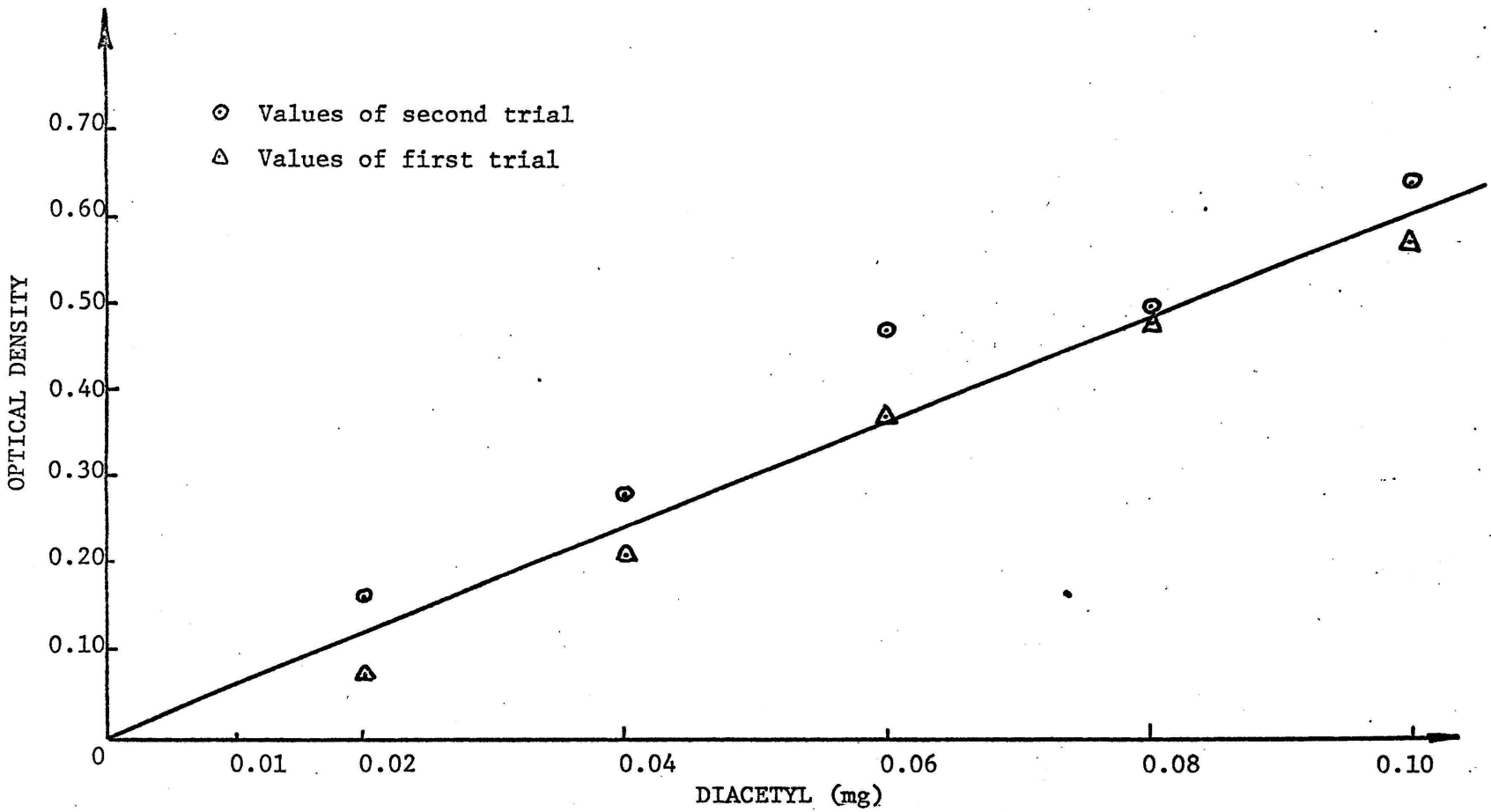


Figure 6. Standard curve for diacetyl determination by a modified Prill and Hammer method.

The diacetyl values found for the ten lots of dairy spread when they were freshly prepared and again after they had been stored for 90 days are shown in Table 4.

TABLE 4

Diacetyl in dairy spread
as determined by Prill and Hammer method as modified by Alfke (1).

Lot no.	Diacetyl ppm	
	fresh	90 days
346	1.40	2.96
347	1.60	2.48
348	1.23	1.41
349	1.56	2.75
350	1.57	2.79
351	1.43	2.83
352	1.41	0.08
353	1.53	2.25
354	1.35	0.91
355	1.49	0.96

Since the flavoring ingredients were added to the product at almost the same temperature after the pasteurization and before the homogenization steps, the results showed very similar diacetyl levels in all ten lots of the dairy spread initially.

After 90 days the results showed an increase in diacetyl content

in almost all the samples. This coincided with a higher oxidoreduction potential. On the other hand some samples with decreased diacetyl content as lots 354 and 355, presented also lower redox potential after 90 days. The diacetyl value for lot 352 after 90 days was considered as inaccurate because of a laboratory accident. These figures seemed to put in evidence some relation between the oxidation-reduction potential and the equilibrium of the chemical reaction of the diacetyl which can shift to acetylmethylcarbinol or 2, 3-butylene glycol in reduction and oxidation reaction, respectively. The latter compounds do not have flavoring properties (80).

Ascorbic Acid Determination.

For standardization of the dye solution, 5 ml of freshly prepared standard ascorbic acid solution (containing 0.5 mg), plus 15 ml 0.1 N H_2SO_4 and 15 ml distilled water was titrated at once with the dye solution. Each of duplicate titrations required 3.70 ml of the dye solution to yield a pink color stable during 30 seconds. A blank titration on the acid-water mixture to achieve the same color and stability consumed 0.10 ml of the dye solution. Hence, the milligrams of ascorbic acid per milliliter of dye was $\frac{0.5 \text{ mg}}{3.70 - 0.10}$ or 0.1388 mg/ml.

Duplicate 12 g samples of every lot (with the exception of lot no. 353) of dairy spread were dissolved in distilled water at 45°C. They were made up to 100 ml then 25 ml of 0.1 N H_2SO_4 were added and the total volume titrated with 2, 6-dichlorophenolindophenol until the pink color remained 30 seconds to several minutes. Because of its purposely-increased content of ascorbic acid, the sample of lot 353 was pre-

pared with two grams of dairy spread in 125 ml total acid-water solution, and only 10 ml of this solution were titrated.

Blanks were prepared in two ways which gave similar results. Three samples were allowed to stand in a cooler for 5 days and another 3 samples were exposed to sunlight until constant low readings of ascorbic acid were reached. The readings were very similar and averaged 0.4 ml. This average value was used in the subsequent calculations.

Table 5 illustrates the results obtained in the titrations and the milligrams of ascorbic acid in the dairy spread. The formula used for calculation was:

$$\text{Ascorbic acid mg/100 g} = \frac{(\text{ml dye-ml blank}) \times 0.1388 \text{ mg/ml} \times 100}{\text{sample g.}}$$

The lowest values were obtained in lots 349 and 350 which had the addition of lactic acid, which lowered the pH of the fresh dairy spread to pH 5.30 and pH 5.65 respectively. The highest value corresponded of course to lot 353 to which purposely 15 grams of ascorbic acid were added.

The suitability of Sharp's method to this new application to dairy spread was checked by adding known amounts of ascorbic acid to freshly prepared dilutions of dairy spread. Samples were titrated before and after the addition of the extra ascorbic acid, with 2, 6-dichlorophenolindophenol and by difference very close values were obtained to those actually added.

By the application of this method, for example, a sample of lot 353 gave a quite reasonable value of ascorbic acid. The calculated

TABLE 5

Ascorbic acid in dairy spread when freshly prepared

Lot no.	Sample, g	Dye, ml	Blank ml	Ascorbic acid mg/100 g
346	12	1.20	0.4	0.925
347	12	1.30	0.4	1.041
348	12	1.00	0.4	0.694
349	12	0.80	0.4	0.462
350	12	0.70	0.4	0.347
351	12	1.20	0.4	0.925
352	12	1.10	0.4	0.810
353	2	1.65	0.4	108.437
354	12	1.50	0.4	1.272
355	12	1.20	0.4	0.925

amount of ascorbic acid was 108.43 mg per 100 grams of dairy spread. The total weight of the batch was 13,582.45 grams; therefore the calculated amount per 100 grams times the weight of the batch divided by 100 was 14.73 grams of ascorbic acid. Considering that the pasteurization process should have eliminated some amount of the vitamin, the above figure may be considered fairly good, since it was close to the 15 grams of ascorbic acid actually added.

When values for the ascorbic acid content were compared with the oxidation-reduction potential values; in general there

was observed in the fresh product an inverse relationship between these two factors. The lots having the lowest ascorbic acid contents exhibited the highest oxidation-reduction potentials, and vice versa.

Electrophoresis Applied to Dairy Spread

The first electrophoretic trials in this study were done with vertical starch gel electrophoresis and were performed on commercial milk casein and dairy spread samples, which were diluted in some trials in the same Tris-citric buffer used for the starch gel preparation. However when concentrated (7 M) urea buffer was used as diluent in the sample preparation the sharpness of the resulting spots was increased.

Milk proteins at pH 8.6 were negatively charged and consequently they migrated to the anode connected to the lower electrode chamber.

The DC electric current was supplied by a Buchler Power Unit No. 3-1014A for constant voltage or constant current operation regulated to a maximum of 1000 VDC and 200 milliamperes.

Most of the trials were performed at constant voltage operation between 80 and 120 volts; and the amperage was balanced automatically, with the variation of the gel resistance causing the readings to decrease from approximately 20 mA at the starting time to 5 mA at the end of the run after 15 to 17 hours. A brownish front line was always formed and it travelled toward the positive pole. It was useful in that at any moment the position of the fastest protein in the gel could be perceived.

Casein samples resulted in three definite spots corresponding

to α-casein, the fastest; β-casein; and γ-casein, the slowest, as reported by Mellander (67).

Dairy spread samples developed spots at the same migration rate as α, β, and γ-casein but they were overlapped with milk serum proteins (16). Different voltages, amperages, samples concentrations, running times, and buffer solutions were tested in order to separate the spots. Resolution was improved but there was not definite isolation such as was obtained in the acrylamide gel electrophoresis developed at the same time.

Polyacrylamide Vertical Gel Electrophoresis.

Polyacrylamide gel columns were prepared as indicated in the experimental procedure section.

Samples were prepared to contain approximately 5 milligrams of dairy spread dry matter per milliliter of solution. For this purpose, 5 g of dairy spread were dissolved in 250 ml boric acid-tetraborate buffer solution of pH 8.2 (the same buffer solution that was used in the electrodes chambers). From this solution one milliliter, containing 20 mg of dairy spread, was mixed with 1.1 ml of solution no. 5 and one drop each of solutions no. 3 and no. 4. The resulting concentration was 9.1 mg/ml of whole sample or approximately 5 mg of dairy spread dry matter per milliliter. Two milliliters of sample were used in each column for a total of about 10 mg of dry matter sample.

Constant voltage was used in the electrophoretic run. Good results were obtained with 100 volts when all eleven acrylamide columns were used. The starting current was in the range of 33 to 35 mA

which made an average of about 3 mA for each column. Runs of 17 hours gave good resolutions. The amperage decreased gradually from the initial 35 mA to 20 mA at the end of the run.

Extraction of the gel columns, fixation, staining and destaining procedures followed the electrophoresis run as described previously.

Commercial casein submitted to acrylamide vertical gel electrophoresis yielded three definite spots as in the starch gel electrophoresis. They corresponded to α, β, and γ casein. The compound with the greater mobility was α-casein and also it was stained the least intensely of the three as shown in Figure 7, sample B.

The analysis of the spots obtained from a fresh dairy spread product, as lot 346, revealed the presence of at least six of the eight components of the milk proteins that have been isolated before by other workers with the aid of the electrophoretic method (17, 35, 60, 86, 106). These eight components as reported by Brunner et al. (16) were: α-casein, β-casein, γ-casein, β-lactoglobulin, α-lactalbumin, blood serum albumin, euglobulin and pseudoglobulin. Table 6 extracted from Brunner as cited by Gordon and Whittier (35) shows their approximate percentage in skimmilk protein and their molecular weights. For comparison purposes the proteins were placed in order according to their approximate percentage of skimmilk protein.

In electrophoretic runs the linear displacement of a boundary from the starting point, the time, and the voltage drop across the gel are used to calculate the mobility of the protein which is expressed as centimeters per second per unit change in electrical field strength (volt/cm) or $cm^2 volts^{-1}\ sec^{-1}$.

TABLE 6

Major milk proteins,
approximate percentage of skimmilk proteins, and molecular weight

Protein	Approx. %	Mol. wt.
1- α-casein	45-63	121,800
2- β-casein	19-28	24,100
3- β-lactoglobulin	7-12	35,000
4- γ-casein	3-7	30,600
5- α-lactalbumin	2-5	15,500
6- Euglobulin	0.8-1.7	180,000
7- Pseudoglobulin	0.6-1.4	180,000
8- Blood serum albumin	0.7-1.3	69,000

The same milk proteins were placed in order according to their electrophoretic mobility at pH 8.6 in veronal buffer, ionic strength = 0.1, starting from the fastest α-casein (16).

By comparing the mobilities shown in Table 7 and the percentages in Table 6 with the results of fresh dairy spread electrophoresis in Figure 7, sample A,it could be concluded that the protein fractions present in dairy spread when freshly produced were then shown in Table 8 in which the identifications "a" to "f" corresponded to those shown in sample A of Figure 7.

The identity of the proteins listed in Table 8 was supported by the proximity of the electrophoretic mobility values to those reported by Brunner et al.(16) for these respective proteins and the

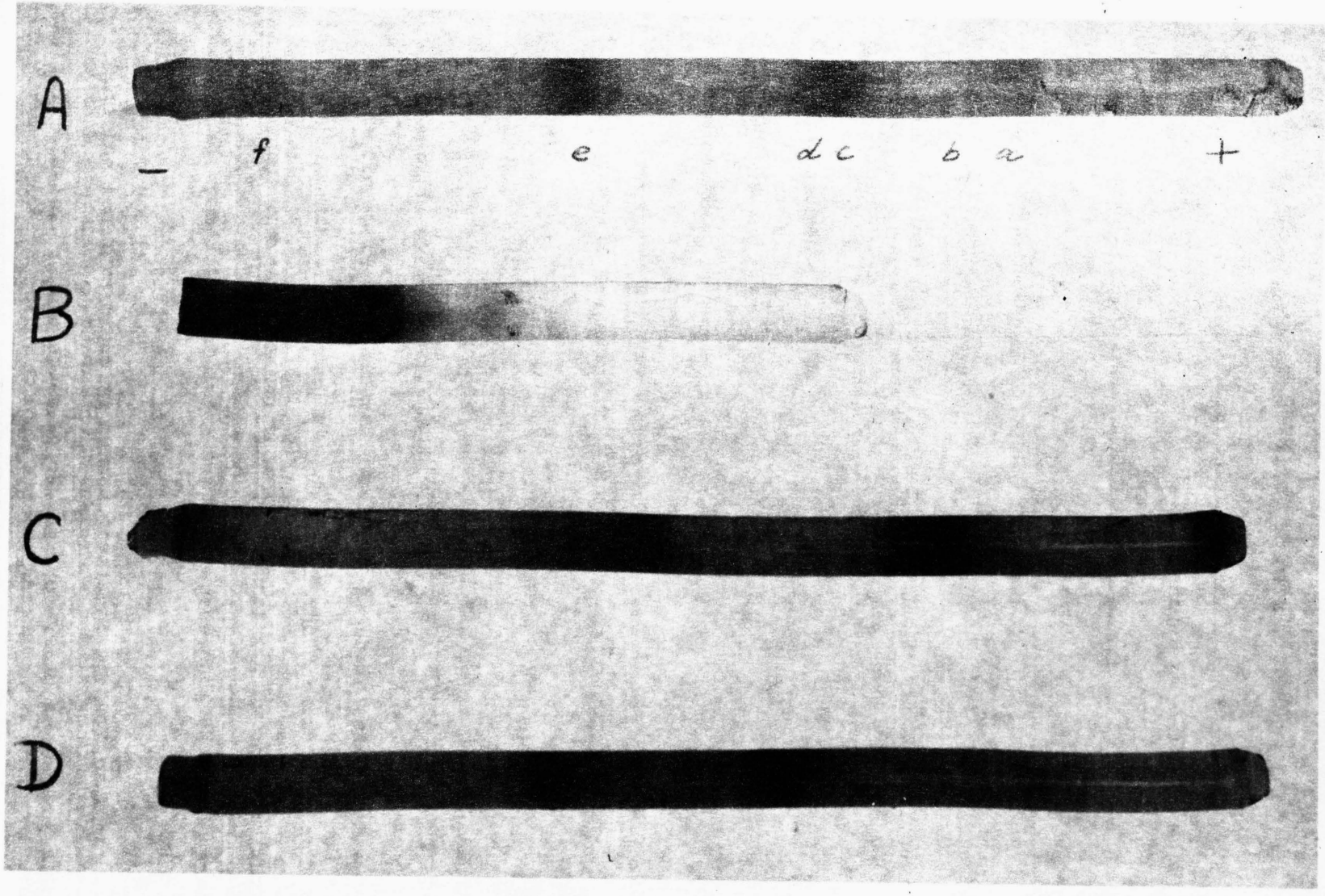


Figure 7. Migration of dairy spread proteins on polyacrylamide vertical gel columns electrophoresis.

TABLE 7

Electrophoretic mobility of milk proteins

Protein	Isoelectric point	Mobility *
1- α-casein	4.1	-6.7
2- Blood serum albumin	4.7	-6.7
3- β-lactoglobulin	5.1	-5.1
4- α-lactalbumin	5.1	-4.2
5- β-casein	4.5	-3.1
6- Pseudoglobulin	5.6	-2.2
7- γ-casein	5.8	-2.0
8- Euglobulin	6.0	-1.8

* Electrophoretic mobility at pH 8.6 in veronal buffer, ionic strength = 0.1

relationship between the color intensity of the spots and the percentages present in skimmilk protein.

When the spots were ordered according to their color intensity and named following the Table 6 order,with the darkest spot as β-casein (α-casein was the exception; it had less intensity as in the electrophoresis of commercial casein) a perfect correspondence was obtained with the electrophoretic mobilities listed in Table 7.

All the ten lots of dairy spread developed a similar electrophoretic display. The changes in pH, or processing temperatures, or

TABLE 8

Milk proteins in fresh dairy spread
as found in
acrylamide vertical gel electrophoresis *

Position in Fig. 7	Protein	Distance travelled in mm	Electro-phoretic mobility	Order of color intensity
a.	α-casein	158	-6.8	--
b.	Blood serum albumin	152	-6.5	5th
c.	β-lactoglobulin	130	-5.6	2nd
d.	α-lactalbumin	125	-5.2	4th
e.	β-casein	78	-3.3	1st
f.	γ-casein or Euglobulin	18	-1.2	3rd

* Buffer solution: Boric acid-Tetraborate, pH 8.6
Running time : 16 hours
Voltage : 100 volts (constant). Field strength: 4 volts/cm
Amperage : 35 mA (variable)

changes in composition did not affect the presence of the six milk proteinic groups.

After a storage period of two months at 40°F all the lots were submitted to electrophoretic analysis again and the results showed a quite different aspect. Some of the proteins disappeared. The only ones found were α-casein, β-lactoglobulin and β-casein, as can be seen in Figure 7, sample C, and they were identified by their electrophoretic mobilities.

After three and a half months, α-casein also disappeared leaving only β-lactoglobulin and β-casein (Figure 7, sample D).

It was evident that a progressing proteolytic activity took place during the storage time. The high molecular weight proteins were split gradually to proteoses, peptones, amino acids and perhaps ammonia in some cases. The Biuret reaction, specific for the peptide linkage, was performed with the aged products and gave a very weak positive reaction in some samples and negative in others. This indicated the breakdown of the peptide chains into shorter ones. The Biuret reaction with fresh dairy spread gave strongly positive result.

The Ninhydrin test also was performed. This test is specific for the free α-amino groups, which increase in number as the long peptide chains are split into shorter compounds. The fresh product gave a weak positive reaction while these was a strong positive on the aged ones.

Thus, both reactions, Biuret and Ninhydrin test, confirmed the supposition that proteolysis had occurred.

The fact that the products of the proteolysis did not appear in the acrylamide gel electrophoresis was attributed to the concentration of the gel which determined the size of the pores. Since high concentration resulted in small pore size so that large molecules could not penetrate the gel and vice versa, smaller molecules would have had greater mobility. It was possible that the degradation products, with smaller molecules, had their mobilities largely increased and they would have run out of the column into the anode buffer solution. This supposition was supported by the results of another trial with dairy spread and single amino acids in the same electrophoretic run. After

15 hours at 100 volts and 35 mA. the amino acids spots did not appear with the regular staining. The specificity or concentration of the gel, or application time of the stain, apparently were not correct for determining amino acids and, perhaps, short peptides.

TABLE 9

Dairy spread microbiological counts *

Lot no.	Standard plate count Organisms per gram			Yeasts and molds Organisms per gram		
	fresh	30 days	60 days	fresh	30 days	60 days
346	1250	7750	400	4	11	0
347	4000	8200	2500	0	9	0
348	310	4250	500	0	3	0
349	280	1850	2600	5	6	0
350	600	4350	3000	0	9	10
351	250	4700	3500	0	7	0
352	600	750	700	0	2	0
353	260	1500	7000	3	1	12
354	1700	3650	1500	0	4	10
355	500	7300	3000	0	5	2

* All coliform plates were negative.

TABLE 10

Protein, fat, and solids, contents, and pH of dairy spread samples freshly produced and after 90 days storage at 38°F.

Lot no.	Proteins %		pH		Fat %		Solids %	
	fresh	90 days	fresh	90 days	fresh	90 days	fresh	90 days
346	5.39	5.31	6.05	7.45	43.7699	45.2929	57.8794	58.2003
347	5.63	5.63	6.10	7.25	43.8524	44.6571	58.4391	58.5616
348	5.59	5.48	5.90	7.40	44.9901	45.6698	57.8252	59.8008
349	5.36	5.62	5.40	7.40	43.7905	43.9864	58.0290	57.2383
350	4.92	5.44	5.65	7.20	44.4827	42.5121	56.9909	56.8688
351	5.14	5.50	6.00	7.60	44.1137	44.8289	58.8638	56.4593
352	5.61	5.70	6.10	7.60	42.8898	43.5072	56.0482	56.0960
353	5.03	5.33	5.75	7.55	41.3611	42.0130	55.6763	56.7373
354	5.28	5.04	6.10	7.65	43.2653	43.4801	56.8887	55.6184
355	5.49	5.55	6.00	7.70	44.4258	43.3371	58.3388	58.1706

SUMMARY AND CONCLUSIONS

Ten lots of dairy spread with variations in their composition or processing were made to study the effect of these variables on the physico-chemical equilibria and shifts therein during storage which determined the characteristics of the body, texture and flavor.

Various analytical approaches were used to study the oxidizing capacity of protein sulfhydryl groups; oxidation-reduction potential; diacetyl content; ascorbic acid; protein degradation; pH levels; and microbiological quality.

Protein Sulfhydryl Groups

1. Pasteurization of dairy spread with standard formula composition at 165°F for 30 minutes apparently continued the protein denaturation, already started in the drying process of the nonfat dry milk, with the subsequent uncoiling of their helix structure, and increased the sulfhydryl group reactivity, which was measured by amperometric titration with iodine and o-iodosobenzoate.
2. Pasteurization of dairy spread with standard formula composition at 175°F for 30 minutes seemed to decrease the sulfhydryl groups reactivity. Apparently there was a "critical temperature" point over 165°F and below 175°, where the activation of the sulfhydryl groups ceased and then began to decrease. This phenomenon followed the conclusions of Zweig and Block (109), who reported that the loss of sulfhydryl groups was a function of time and temperature when the latter was above the "critical

temperature", and that once that point was reached the concentration of sulfhydryl groups decreased very rapidly with the increase of the temperature. These authors stated that "Cooked" flavor appeared and volatile sulfides were evolved at the critical point.

3. Addition of lactic acid to the regular dairy spread mix before the pasteurization lowered the pH from 6.05 to 5.6 and 5.3 in two batches, respectively. The concentration of sulfhydryl groups was lowered approximately to the same amount that higher pasteurization temperatures had produced. Presumably the presence of lactic acid affected the extent of denaturation of the proteins by heat treatment.
4. When condensed skimmilk was used as the source of milk solids for dairy spread there was less sulfhydryl group reactivity compared with the activity in standard batches.
5. Addition of ascorbic acid to the regular dairy spread mix before its pasteurization resulted in less sulfhydryl reactivity than was in standard dairy spread but more than when the above mentioned treatments were applied. The pH of the batch was lowered to pH 5.7.
6. The use of high heat nonfat dry milk as milk solids source brought about very low reactivity of the sulfhydryl groups. The higher temperature treatment of this type of milk powder apparently released the free sulfhydryl groups from the denatured protein and volatilized them (109).
7. Air oxidation seemed to cause a decrease of free sulfhydryl

group concentration. Intensifying the air oxidation of a standard dairy spread mix by vigorous recirculation through a centrifugal pump during the pasteurization gave 40% less reactivity of the sulfhydryl groups. Larson and Jenness (58) reported that probably the decrease of reducing capacity following treatment at higher temperature is due to oxidation since it was largely prevented by excluding air from the samples during heating and replacing it with nitrogen.

Oxidation-Reduction Potential

8. Standard batches of dairy spread presented a constant level of redox potential which was maintained for 60 days and started to increase after the second month which coincided with changes in the body and texture.

9. Dairy spread processed with higher pasteurization treatments (175°F and 178°F) seemed to have reduced oxidation-reduction potential in the fresh product. Several researchers stated that the lowering of the redox potential coincided with the liberation of the sulfhydryl groups (30, 41, 71).

   The lower redox potential was not maintained at the same level during storage; it increased rapidly between 30 and 60 days storage at 38°F.

10. Use of unpasteurized cream as a fat source for dairy spread preparation processed at standard pasteurization treatment (165°F for 30 minutes) gave a slightly lower initial redox potential, which level was lower than all the above batches even at 90 days of storage.

11. Ascorbic acid added to dairy spread mix before pasteurization (about 110 mg per 100 g dairy spread) produced a markedly lower oxidation-reduction potential, which was lowered even more during 90 days storage at 38°F.

12. The dairy spread lot having high heat nonfat dry milk in its composition exhibited a lower oxidation-reduction potential in the fresh product. The potential decreased gradually after 30 days of storage but not so low as ascorbic acid had promoted in the precedent batch. The lower potential in this batch coincided with the total liberation of protein sulfhydryl groups.

13. Increased air oxidation by intense recirculation of standard dairy spread mix during pasteurization at 165°F for 30 minutes gave the higher redox potential in the fresh product. Apparently the natural ascorbic acid content which is the most important factor in milk redox potential was increasing the potential according to the fundamental formula used in this paper (52, 70). Upon storage, a possible hydrolysis, after 30 days, of the dehydroascorbic acid to diketogulonic acid, favored by the observed increase of pH, decreased gradually the redox potential again.

Diacetyl

14. After 90 days of storage most of the samples were found to have an increase of the diacetyl content. This paralleled the increase of the oxidation-reduction potential in the same samples. The samples which displayed a decrease in diacetyl content presented also lower redox potential after 90 days of storage. Presumably the redox potential has an influence in the equilibria

among diacetyl, acetylmethylcarbinol, and 2, 3-butyleneglycol. Diacetyl is converted to one of the other compounds by reduction or oxidation, respectively. Since the last two compounds contributed no flavor or aroma, the shifts in the respective quantities of the compounds distinctly affected the flavor of the spread.

Ascorbic Acid

15. In general, an inverse relationship was observed between the ascorbic acid content and oxidation-reduction potential in all the samples. It seemed that the ascorbic acid had a definite influence on the redox potential.

Protein Degradation

16. Electrophoretic mobility patterns in polyacrylamide vertical gel electrophoresis, and the relative protein percentages as indicated by intensity of staining and width of band demonstrated at least six of the eight known and isolated milk proteins reported by Brunner et al.(16). The proteins isolated were presumed to be α-casein, blood serum albumin, β-lactoglobulin, α-lactalbumin, β-casein, and either γ-casein or euglobulin.

17. Aged dairy spread samples following storage at 38°F for 60 and 90 days, seemed to have undergone a progressive proteolysis. Proteins were broken down to shorter chains as indicated by Ninhydrin test, Biuret reaction, and electrophoretic runs. The latter demonstrated progressively fewer proteins as the storage time became longer.

Others

18. Total nitrogen content by Kjeldahl procedure remained almost constant. (Table 10).
19. Solids and fat remained at essentially the same level; variations could be attributed to changes in the moisture content. (Table 10).
20. All the samples increased in pH values as storage progressed. (Table 10).
21. Bacteriological counts presented very low figures, which increased moderately upon storage. Low counts of yeast and molds and no coliform were present in fresh and aged product. (Table 9).
22. An interrelationship seemed to exist among the redox potential, ascorbic-dehydroascorbic acid equilibrium and the free sulfhydryl groups content which imparted definite characteristics to the body, texture, and flavor of the tested samples.
23. Redox potentials and electrophoretic runs showed that dairy spread had a shelf life of about 60 days with good keeping quality when stored at 38°F.
24. Air oxidation and high heat nonfat dry milk did not favor the keeping quality of dairy spreads. They lowered the redox potential which seemed to promote formation of more diacetyl; however, non pleasant flavoring compounds, which perhaps originated in the protein hydrolysis, were also produced.
25. Addition of extra ascorbic acid to dairy spread mixes did not help to improve the keeping quality.
26. Higher pasteurization temperatures helped in the initial redox potential and the keeping quality.

BIBLIOGRAPHY

1. Alfke, B. D. 1967. A Modified Prill and Hammer Method Used in the Study of Diacetyl Production by Single Strain Lactic Streptococci. M.S. Thesis, South Dakota State University, Brookings, South Dakota.

2. Anson, M. L. 1940. The Reaction of Iodine and Iodoacetamide with Native Egg Albumin. J. Gen. Physiol., 23:321-331.

3. Anson, M. L. 1945. Protein Denaturation and the Properties of Protein Groups, p. 361-384. In M. L. Anson and J. T. Edsall (ed) Advances in Protein Chemistry. Academic Press, Inc., New York. Cited in Webb and Johnson (98).

4. Bailey, J. L. 1967. Techniques in Protein Chemistry. Elsevier Publishing Co., New York. 406 p.

5. Barron, E. S. G. and Hastings, A. B. 1934. III The Oxidation-Reduction Potential of the System Lactate-enzyme-pyruvate. J. Biol. Chem., 107:567.

6. Beckman Instruments. 1959. Scientific and Process Instruments Division. Application Data Sheet: GS-44C.

7. Benesch, R. E., Lardy, H. A., and Benesch, R. 1935. J. Biol. Chem., 212:235. Cited in West, Todd, Mason and Bruggen, (105) p. 297.

8. Bloemendahl, H. 1963. Zone Electrophoresis in Blocks and Columns. Elsevier Publishing Co., New York. Cited in Boyer (10).

9. Boyer, P. D. 1954. J. Amer. Chem. Soc., 76:4331. Cited in West, Todd, Mason and Bruggen (105) p. 297.

10. Boyer, S. H. 1964. Instructions for Starch Gel Electrophoresis. Buchler Instruments, Inc. Fort Lee, New Jersey. 19 p.

11. Boyer, S. H. and Hiner, R. S. 1963. Lab. Clin. Med., 61:879. Cited in Boyer (10).

12. Briggs, D. R. and Hull, R. 1945. Studies on Protein Denaturation. I. Electrophoretic Study Kinetics at Neutrality of Heat Denaturation of β-Lactoglobulin. J. Amer. Chem. Soc., 67:2007.

13. Bugai, P. M. 1938. Casein, a Mixture of Several Proteins. Chem. Abstr., 32:3840.

14. Bullock, D. H. January, 1966. A Preliminary Study of a New Low-Fat Dairy Spread. Canadian Dairy and Ice Cream Journal, 45:26.

15. Brunner, J. R. 1965. Physical Equilibria in Milk: The Lipid Phase. p. 403. In B. H. Webb and A. H. Johnson (ed). Fundamentals of Dairy Chemistry. The Avi Publishing Company, Inc., Westport, Connecticut.

16. Brunner, J. R., Ernstrom, C. A., Hollis, R. A., Larson, B. L., Whitney, R. Mc L., and Zittle, C. A. 1960. Nomenclature of the Proteins of Bovine Milk. First Revision. J. Dairy Sci., 43:901.

17. Brunner, J. R. and Thompson, M. P. 1961. Characteristics of Several Minor-Protein Fractions Isolated from Bovine Milk. J. Dairy Sci., 44:1224.

18. Campbell, J. J. R., Phelps, R. H., and Keur, L. B. 1959. Dependence of Oxidation-Reduction Potential of Milk on its Vitamin C Content. J. Milk Food Technol., 22:346.

19. Carlson, L. A. 1954. Electrophoretic Studies of Serum Lipoproteins. I. A Description of Apparatus and Technique for their Separation in Starch Medium. Acta Chem. Scand., 8:510.

20. Cecil, R. 1964. p. 379. In H. Neurath (ed). The Proteins, 2nd ed., Vol. I. Cited in West and Todd (105).

21. Cherbuliez, E., and Schneider, M. L. 1932. Helv. Chim. Acta, 15:597. Cited by Gordon and Whittier (35).

22. Clarck, W. M. and Cohen, B. 1923. U. S. Public Health Reports, 38:666. Cited by Gordon and Whittier (35).

23. Clarck, W. M. and Cohen, B. 1928. Hygienic Laboratory Bull. 151:13. Cited by Gordon and Whittier (35).

24. Cooper, G. R. 1960. The Plasma Proteins. Vol. I Academic Press, Inc., New York. Cited in Boyer (10).

25. Coulter, S. T., Jenness, R., and Geddes, W. F. 1951. Physical and Chemical Aspects of the Production, Storage and Utility of Dry Milk Products. In E. M. Mrak and G. F. Steward (ed). Advances in Food Research 3:45. Academic Press Inc., New York.

26. Crowelk, L. K., Jenness, R., and Coulter, S. T. 1948. The Reducing Capacity of Milk and Milk Products as Measured by a Modified Ferricyanide Method. J. Dairy Sci., 31:595.

27. Crowther, C. and Raistrick, H. 1916. A Comparative Study of the Proteins of the Colostrum and Milk of the Cow and their Relations to Serum Proteins. Biochem. J., 10:434.

28. Deutsch, H. F. 1947. A Study of Whey Proteins from the Milk of Various Animals. J. Biol. Chem., 169:437.

29. Doan, F. J., and Josephson, D. V. 1943. Observation on the Ascorbic Acid Content of Evaporated Milk. J. Dairy Sci., 26:1031.

30. Eilers, H., Saal, R. N. J., and van der Waarden, M. 1947. Chemical and Physical Investigations of Dairy Products. Elsevier Publishing Co., New York. Cited in R. Jenness and S. Patton. 1959. Principles of Dairy Chemistry. John Wiley and Sons, Inc., New York.

31. Fine, I. H. and Costello, L. A. 1963. In S. P. Colowick and N. O. Kaplan (ed). Methods of Enzymology, 6:958. Academic Press, New York.

32. Flodin, P. and Porath, J. 1954. Zone Electrophoresis in Starch Columns. Biochem. et. Biophys., 13:175.

33. Foulk, C. W. and Bawden, A. T. 1926. A New Type of Endpoint in Electrometric Titration and its Application to Iodometry. J. Amer. Chem. Soc., 48:2045.

34. Frazier, W. C. and Whittier, E. O. 1931. Studies on the Influence of Bacteria on the Oxidation-Reduction Potential of Milk. I. Influence of Pure Cultures of Milk Organisms. J. Bact., 21:239.

35. Gordon, W. G. and Whittier, E. O. 1965. Proteins of Milk, p. 54-90. In B. H. Webb and A. H. Johnson (ed) Fundamentals of Dairy Chemistry. The Avi Publishing Company, Inc., Westport, Connecticut.

36. Grelck, W. P. M. July 23, 1935. U. S. Patent 2,009,135. Cited in Whittier and Webb (107).

37. Grelck, W. P. M. July 23, 1935. U. S. Patent 2,009,136. Cited in Whittier and Webb (107).

38. Haglund, H. and Tiselius, A. 1950. Zone Electrophoresis in a Glass Powder Column. Acta Chem. Scan., 4:957.

39. Hall, C. W. and Hedrick, T. I. 1966. Drying Milk and Milk Products. p. 140. The Avi Publishing Company, Inc., Westport, Connecticut.

40. Harland, H. A., Coulter, S. T., and Jenness, R. 1949. Some Factors Influencing the Reducing Systems in Dry Whole Milk. J. Dairy Sci., 32:334.

41. Harland, H. A., Coulter, S. T., and Jenness, R. 1952. The Interrelationships of Processing Treatments and Oxidation-Reduction Systems as Factors Affecting the Keeping Quality of Dry Whole Milk. J. Dairy Sci., 35:643.

42. Hartman, A. M., and Dryden, L. P. 1965. The Vitamins in Milk and Milk Products. p. 314 in B. H. Webb and A. H. Johnson (ed) Fundamentals of Dairy Chemistry. The Avi Publishing Co., Westport, Connecticut.

43. Hellerman, L., Chinard, F. P., and Deitz, D. R. 1943. Protein Sulfhydryl Groups and the Reversible Inactivation of the Enzyme Urease. The Reducing Groups of Egg Albumin and Urease. J. Biol. Chem., 147:443.

44. Hellerman, L., Chinard, F. P., and Ramsdell, P. A. 1941. o-Iodosobenzoic Acid. A Reagent for the Estimation of Cysteine, Glutathione and the Substituent Sulfhydryl Groups of Certain Proteins. J. Amer. Chem. Soc., 63:2551.

45. Hess, W. C. and Sullivan, M. S. 1943. The Cysteine, Cystine and Methionine Content of Proteins. J. Biol. Chem., 151:365-642.

46. Hutton, J. T. and Patton, S. 1952. The Origin of Sulfhydryl Groups in Milk Proteins and their Contribution to "Cooked Flavor". J. Dairy Sci., 35:699.

47. Jackson, C. J. 1936. Factors in the Reduction of Methylene Blue in Milk. J. Dairy Res., 7:31.

48. Jenness, R. 1954. Milk Proteins.Effect of Heat Treatment on Serum Proteins. J. Agr. Food Chem., 2:75.

49. Jenness, R. and Patton, S. 1955. Milk Proteins, p. 112. In R. Jenness and S. Patton, Principles of Dairy Chemistry. John Wiley and Sons, Inc., New York.

50. Jenness, R. and Patton, S. 1959. The Effects of Heat on Milk. p. 336. In R. Jenness and S. Patton, Principles of Dairy Chemistry. John Wiley and Sons, Inc., New York.

51. Jenness, R. and Patton, S. 1959. Flavors and Off-Flavors in Milk. p. 385. In R. Jenness and S. Patton, Principles of Dairy Chemistry. John Wiley and Sons, Inc., New York.

52. Jenness, R., Shipe, W. F., Jr. and Whitnah, C. H. 1965. Physical Properties of Milk Oxidation-Reduction Equilibria. p. 345 in B. H. Webb and A. H. Johnson, Fundamentals of Dairy Chemistry. The Avi Publishing Company, Inc., Westport, Connecticut.

53. Josephson, D. V. and Doan, F. J. 1939. Observations on Cooked Flavor in Milk. Its Sources and Significance. Milk Dealer, 29:35.

54. Kunkel, H. G. 1951. Methods of Biochemical Analysis. Vol I. Interscience. New York. Cited in Boyer (10).

55. Kunkel, H. G. and Slater, R. J. 1952. Proc. Soc. Exp. Biol. Med., 80:42. Cited in Boyer (10).

56. Larsen, R. A., Jenness, R. and Geddes, W. F. 1949. Effect of Heat Treatment on the Sulfhydryl Groups of Milk Serum Proteins. Cereal Chem., 26:287.

57. Larson, B. L. and Jenness, R. 1950. The Determination of Protein Sulfhydryl Groups with Iodine and o-Iodosobenzoate by Amperometric Titration. J. Dairy Sci., 33:890.

58. Larson, B. L. and Jenness, R. 1950. The Reducing Capacity of Milk as Measured by an Iodimetric Titration. J. Dairy Sci., 33:896.

59. Larson, B. L. and Jenness, R. 1952. Characterization of the Sulfhydryl Groups and the Kinetics of the Heat Denaturation of Crystalline β-Lactoglobulin. J. Amer. Chem. Soc. 74:3090.

60. Larson, B. L. and Jenness, R. 1955. Identification of α-Lactalbumin in the Electrophoretic Pattern of Milk Serum Proteins. J. Dairy Sci., 38:313.

61. Larson, B. L. and Rolleri, G. D. 1955. Heat Denaturation of the Specific Serum Proteins in Milk. J. Dairy Sci., 38:351.

62. Leopold, O. June 24, 1930. U. S. Patent 1,767,083. Cited in Whittier and Webb (107).

63. Light, A. and Smith, E. L. 1963. Page 1. In H. Neurath (ed). The Proteins, 2nd ed. Vol. I. Academic Press, New York. Cited in West, Todd, Mason and Bruggen (105) p. 297.

64. Linderstrom-Lang, K. 1929. Compt. Rend. Trav. Lab. Carlsberg. 17 No. 9. Cited by Hartman and Dryden (42).

65. Linderstrom-Lang, K. and Kodama, S. 1925. Compt. Rend. Trav. Lab. Carlsberg. 16 No. 1. Cited by Hartman and Dryden (42).

66. Mapson, L. W. 1961. The Estimation of Dehydro-L-Ascorbic Acid when Present in Low Concentration in Tissues, by the Roe and Kuether Procedure. Ann. N. Y. Acad. Sci., 92:284.

67. Mellander, O. 1939. Electrophoretische Untersuchung von Casein. Biochem. Z., 300:240.

68. Michaelian, M. B., Farmer, R. S., and Hammer, B. W. 1933. The Relationship of Acetylmethylcarbinol and Diacetyl to Butter Cultures. Ia. Agr. Exp. Sta. Res. Bull., 155:322.

69. Michaelian, M. B. and Hammer, B. W. 1935. Studies on Acetylmethylcarbinol and Diacetyl in Dairy Products. Iowa State College of Agriculture and Mechanical Arts Research Bull. 179:203.

70. Michaelis, L. 1951. In J. Sumner and K. Myrback, (ed) The Enzymes, Vol. 2, Part 1. Academic Press, Inc., New York.

71. Neurath, H., Greenstein, J. P., Putnam, F. W., and Erickson, J. O. 1944. The Chemistry of Protein Denaturation. Chem. Rev., 34:157.

72. Ornstein, L. and Davis, B. J. 1962. Disc Electrophoresis, preprinted by Distillation Products, Ind. Eastman Kodak Company.

73. Osborne, T. B. and Wakeman, A. J. 1918. The Proteins of Cow's Milk. J. Biol. Chem., 33:7.

74. Pallavicini, J. C. 1966. Instructions for Polyacrylamide Gel Electrophoresis. Buchler Instruments, Inc. Fort Lee, New Jersey. 8 p.

75. Palmer, A. H. 1939. The Preparation of Crystalline Globulin from the Albumin Fraction of Cow's Milk. J. Biol. Chem., 104:359.

76. Parsons, C. H. September, 1931. U. S. Patent 1,824,496. Cited in Whittier and Webb (107).

77. Porath, J. 1954. Purification of Bacitracin Polypeptides by Charcoal Chromatography and Zone Electrophoresis. Acta Chem. Scan., 8:1813.

78. Porath, J. 1957. Archiv. Uemi II. 161. Cited in Pallavicini (74).

79. Poulik, M. D. 1957. Starch Gel Electrophoresis in a Discontinuous System of Buffers. Nature, 180:1477.

80. Prill, E. A., Fabricius, N. E., and Hammer, B. W. 1939. Diacetyl and Other Alpha-Dicarbonyl Compounds with Special Reference to the Flavor of Butter. Ia. Agr. Exp. Sta. Research Bull. 268:375.

81. Prill, E. A. and Hammer, B. W. 1938. A Colorimetric Method for the Microdetermination of Diacetyl. Iowa State Coll. J. Sci., 12:385.

82. Putnam, F. W. 1953. p. 807. In H. Neurath and K. Bailey (ed). The Proteins. Vol. I. Part B. Academic Press, New York.

83. Raymond, S. and Weintraub, L. 1959. Acrylamide Gel as a Supporting Medium of Zone Electrophoresis. Science, 130:711.

84. Roberts, J. G. 1959. Food Product and Method for Making the Same. U. S. Patent 2,878,126. Cited in Bullock (14).

85. Roe, J. H. 1961. Appraisal of Methods for the Determination of L-Ascorbic Acid. Ann. N. Y. Acad. Sci., 92:277.

86. Rolleri, G. O., Larson, B. L., and Touchberry, R. W. 1956. Protein Production in the Bovine. Breed and Individual Variations in the Specific Protein Constituents of Milk. J. Dairy Sci., 39:1683.

87. Rowland, S. J. 1938. The Precipitation of the Proteins in Milk. I Casein. II Total Proteins. III Globulin. IV Albumin and Proteose-Peptone. J. Dairy Research, 9:30.

88. Sebrell, W. H., Jr. and Harris, R. S. 1954. Editors. The Vitamins, Chemistry, Physiology, Pathology. Vol. 1-3. Academic Press, Inc., New York. Cited in Hartman and Dryden (42).

89. Sharp, P. F. 1938. Rapid Method for the Quantitative Determination of Reduced Ascorbic Acid in Milk. J. Dairy Sci., 21:85.

90. Smith, E. L. 1946. Isolation and Properties of Immune Lactoglobulins from Bovine Whey. J. Biol. Chem., 165:665.

91. Smithies, O. 1955. Zone Electrophoresis in Starch Gels: Group Variations in the Serum Proteins of Normal Human Adults. Biochem. J., 61:629.

92. Smithies, O. 1959. In Advances in Protein Chemistry, Vol. 14. Academic Press, Inc., New York. Cited in Boyer (10).

93. Smithies, O. 1959. An Improved Procedure of Starch-Gel Electrophoresis. Further Variations in the Serum Proteins of Normal Individuals. Biochem. J., 71:585.

94. Stock, J. T. 1948. The Microchemical Aspects of the "Dead Stop" Endpoint Titration Method. Metallurgia, 37:220.

95. Tobias, J. and Tracy, P. H. 1958. Observation on Low Fat Spreads. J. Dairy Sci., 41:1117.

96. Townley, R. C. and Gould, I. A. 1943. A Quantitative Study of Heat Labile Sulfides of Milk. II General Origin of Sulfides and Relation to Total Sulfur, Total Nitrogen, and Albumin Nitrogen. J. Dairy Sci., 26:843.

97. Townely, R. C. and Gould, I. A. 1943. A Quantitative Study of the Heat Labile Sulfides of Milk. III Influence of pH, Added Compounds, Homogenization and Sunlight. J. Dairy Sci., 26:853.

98. Tummerman, L. and Webb, B. H. 1965. Coagulation of Milk and Protein Denaturation. p. 506. In B. H. Webb and A. H. Johnson (ed) 1965 Fundamentals of Dairy Chemistry. The Avi Publishing Company, Inc., Westport, Connecticut.

99. Van Niel, C. G., Kluyver, A. J., and Derx, H. G. 1929. Uber das Butteraroma. Bioch. Ztshr., 210:234. Cited by Michaelian et al (68).

100. Webb, B. H. and Hufnagle, C. F. 1939. Unpublished Results. Cited in Whittier and Webb (107).

101. Weckel, K. G. December, 1943. New Dairy Spreads Developed. Annual Report Agr. Exp. Sta. Univ. of Wisconsin, Bull. 461.

102. Weckel, K. G. June, 1952. Dairy Spreads. Their Potential Market and Manufacturing Procedure. Milk Plant Monthly, 4:24.

103. Weckel, K. G. July, 1965. Dairy Spreads. Manufactured Milk Products Journal, 56:5.

104. West, E. S., Todd, W. R., Mason, H. S., and Van Bruggen, J. T. 1966. Sulfhydryl-Disulfide Exchange Reactions of Peptides and Proteins. p. 335. In E. S. West, W. R. Todd, H. S. Mason, and J. T. Van Bruggen. Textbook of Biochemistry 4th Edition. The Macmillan Company, New York.

105. West, E. S., Todd, W. R., Mason, H. S., and Van Bruggen, J. T. 1966. Determination of Ascorbic Acid, p. 823. In E. S. West, W. R. Todd, H. S. Mason, and J. T. Van Bruggen, Textbook of Biochemistry. 4th Edition. The Macmillan Company, New York.

106. Whitnah, C. H., Rutz, W. M. D., and Fryer, H. C. 1956. Some Physical Properties of Milk. II Effects of Age upon the Viscosity of Pasteurized Whole Milk. J. Dairy Sci., 39:356.

107. Whittier, E. O. and Webb, B. H. 1950. By-Products from Milk. Reinold Publishing Corporation, New York. 317 p.

108. Wilster, G. H. September, 1946. New Things in Dairy Manufacturing. Pac. Dairy Review, 50:9.

109. Zweig, G. and Block, R. J. 1953. The Effect of Heat Treatment on the Sulfhydryl Groups in Skimmilk and Nonfat Dry Milk. J. Dairy Sci., 36:427.

APPENDIX

TABLE 1 A

Amperometric titration of sulfhydryl groups with 0.0005N sodium-o-iodosobenzoate
blank determination

*ml. OIB	+mV.	Δ	ml. OIB	+mV.	Δ
15.0	18.6	-	21.9	33.3	0.0
16.0	18.6	0.0	22.0	33.4	0.1
17.0	18.6	0.0	22.1	33.4	0.0
18.0	18.6	0.0	22.2	33.5	0.1
19.0	18.7	0.01	22.3	33.5	0.0
19.1	18.8	0.1	22.4	33.6	0.1
19.2	19.0	0.2	22.5	33.6	0.0
19.3	19.2	0.2	22.6	33.7	0.1
19.4	19.5	0.3	22.7	33.7	0.0
19.5	20.2	0.7	22.8	33.8	0.1
19.6*	28.0	7.8	22.9	33.9	0.1
19.7	29.6	1.6	23.0	34.0	0.1
19.8	30.5	0.9	23.1	34.0	0.0
19.9	30.9	0.4	23.2	34.0	0.0
20.0	31.1	0.2	23.3	34.1	0.1
20.1	31.4	0.3	23.4	34.1	0.0
20.2	31.7	0.3	23.5	34.2	0.1
20.3	31.9	0.2	23.6	34.2	0.0
20.4	32.1	0.2	23.7	34.2	0.0
20.5	32.3	0.3	23.8	34.2	0.0
20.6	32.4	0.1	23.9	34.3	0.1
20.7	32.5	0.1	24.0	34.3	0.0
20.8	32.6	0.1	24.1	34.3	0.0
20.9	32.7	0.1	24.2	34.3	0.0
21.0	32.8	0.1	24.3	34.4	0.1
21.1	32.9	0.1	24.4	34.4	0.0
21.2	33.0	0.1	24.5	34.4	0.0
21.3	33.0	0.0	24.6	34.5	0.1
21.4	33.1	0.1	24.7	34.5	0.0
21.5	33.1	0.0	24.8	34.5	0.0
21.6	33.2	0.1	24.9	34.6	0.1
21.7	33.2	0.0	25.0	34.6	0.0
21.8	33.3	0.1			

* ml. OIB = milliliters of 0.0005N sodium-o-iodosobenzoate
+mV. = positive millivolts
Δ = +mV. increase between two consecutive readings

TABLE 2 A

Amperometric titration of sulfhydryl groups in dairy spread lot 346, using 0.0005N sodium-o-iodosobenzoate

*ml. OIB	+mV.	Δ	ml. OIB	+mV.	Δ
15.0	17.1	0.0	21.9	17.1	0.0
16.0	17.1	0.0	22.0	17.1	0.0
17.0	17.1	0.0	22.1	17.2	0.1
18.0	17.1	0.0	22.2*	17.8	0.6
19.0	17.1	0.0	22.3	18.2	0.4
19.1	17.1	0.0	22.4	18.2	0.0
19.2	17.1	0.0	22.5	18.3	0.1
19.3	17.1	0.0	22.6	18.4	0.1
19.4	17.1	0.0	22.7	18.5	0.1
19.5	17.1	0.0	22.8	18.6	0.1
19.6	17.1	0.0	22.9	18.7	0.1
19.7	17.1	0.0	23.0	18.8	0.1
19.8	17.1	0.0	23.1	18.9	0.1
19.9	17.1	0.0	23.2	19.1	0.2
20.0	17.1	0.0	23.3	19.2	0.1
20.1	17.1	0.0	23.4	19.3	0.1
20.2	17.1	0.0	23.5	19.5	0.2
20.3	17.1	0.0	23.6	19.6	0.1
20.4	17.1	0.0	23.7	19.7	0.1
20.5	17.1	0.0	23.8	19.8	0.1
20.6	17.1	0.0	23.9	19.9	0.1
20.7	17.1	0.0	24.0	20.0	0.1
20.8	17.1	0.0	24.1	20.2	0.2
20.9	17.1	0.0	24.2	20.3	0.1
21.0	17.1	0.0	24.3	20.4	0.1
21.1	17.1	0.0	24.4	20.5	0.1
21.2	17.1	0.0	24.5	20.6	0.1
21.3	17.1	0.0	24.6	20.7	0.1
21.4	17.1	0.0	24.7	20.8	0.1
21.5	17.1	0.0	24.8	20.9	0.1
21.6	17.1	0.0	24.9	21.0	0.1
21.7	17.1	0.0	25.0	21.1	0.1
21.8	17.1	0.0			

* ml. OIB = milliliters of 0.0005N sodium-o-iodosobenzoate
+mV. = positive millivolts
Δ = +mV. increase between two consecutive readings

TABLE 3 A

Amperometric titration of sulfhydryl groups in dairy spread lot 347, using 0.0005N sodium-o-iodosobenzoate

*ml. OIB	+mV.	Δ	ml. OIB	+mV.	Δ
15.0	18.0	-	21.9	28.5	0.1
16.0	18.0	0.0	22.0	28.6	0.1
17.0	18.0	0.0	22.1	28.7	0.1
18.0	18.0	0.0	22.2	28.8	0.1
19.0	18.0	0.0	22.3	28.9	0.1
19.1	18.0	0.0	22.4	29.0	0.1
19.2	18.1	0.1	22.5	29.1	0.1
19.3	18.5	0.4	22.6	29.2	0.1
19.4	19.0	0.5	22.7	29.3	0.1
19.5	19.4	0.4	22.8	29.4	0.1
19.6	19.8	0.4	22.9	29.5	0.1
19.7	20.4	0.6	23.0	29.6	0.1
19.8	21.6	1.2	23.1	29.7	0.1
19.9*	23.2	1.6	23.2	29.8	0.1
20.0	24.2	1.0	23.3	29.9	0.1
20.1	25.0	0.8	23.4	30.0	0.1
20.2	25.6	0.6	23.5	30.1	0.1
20.3	26.2	0.6	23.6	30.2	0.1
20.4	26.5	0.3	23.7	30.3	0.1
20.5	26.8	0.3	23.8	30.4	0.1
20.6	27.0	0.2	23.9	30.5	0.1
20.7	27.2	0.2	24.0	30.6	0.1
20.8	27.4	0.2	24.1	30.7	0.1
20.9	27.5	0.1	24.2	30.8	0.1
21.0	27.6	0.1	24.3	30.9	0.1
21.1	27.7	0.1	24.4	31.0	0.1
21.2	27.8	0.1	24.5	31.1	0.1
21.3	27.9	0.1	24.6	31.2	0.1
21.4	28.0	0.1	24.7	31.3	0.1
21.5	28.1	0.1	24.8	31.4	0.1
21.6	28.2	0.1	24.9	31.5	0.1
21.7	28.3	0.1	25.0	31.6	0.1
21.8	28.4	0.1			

* ml. OIB = milliliters of 0.0005N sodium-o-iodosobenzoate
+mV. = positive millivolts
Δ = +mV. increase between two consecutive readings

TABLE 4 A

Amperometric titration of sulfhydryl groups in dairy spread lot 348, using 0.0005N sodium-o-iodosobenzoate

*ml. OIB	+mV.	Δ	ml. OIB	+mV.	Δ
15.0	19.5	-	21.9	26.2	0.1
16.0	19.5	0.0	22.0	26.3	0.1
17.0	19.5	0.0	22.1	26.4	0.1
18.0	19.6	0.01	22.2	26.5	0.1
19.0	19.7	0.01	22.3	26.6	0.1
19.1	19.9	0.2	22.4	26.7	0.1
19.2	20.1	0.2	22.5	26.8	0.1
19.3	20.3	0.2	22.6	26.9	0.1
19.4	20.5	0.2	22.7	27.0	0.1
19.5	20.7	0.2	22.8	27.1	0.1
19.6	21.0	0.3	22.9	27.2	0.1
19.7	21.3	0.3	23.0	27.3	0.1
19.8	21.6	0.3	23.1	27.4	0.1
19.9	21.8	0.2	23.2	27.5	0.1
20.0	22.0	0.2	23.3	27.6	0.1
20.1	22.2	0.2	23.4	27.7	0.1
20.2*	23.0	0.8	23.5	27.8	0.1
20.3	23.6	0.6	23.6	27.9	0.1
20.4	23.8	0.2	23.7	28.0	0.1
20.5	24.0	0.2	23.8	28.1	0.1
20.6	24.2	0.2	23.9	28.2	0.1
20.7	24.4	0.2	24.0	28.3	0.1
20.8	24.6	0.2	24.1	28.4	0.1
20.9	24.8	0.2	24.2	28.5	0.1
21.0	24.9	0.1	24.3	28.6	0.1
21.1	25.1	0.2	24.4	28.7	0.1
21.2	25.3	0.2	24.5	28.8	0.1
21.3	25.5	0.2	24.6	28.9	0.1
21.4	25.6	0.1	24.7	29.0	0.1
21.5	25.8	0.2	24.8	29.1	0.1
21.6	25.9	0.1	24.9	29.2	0.1
21.7	26.0	0.1	25.0	29.3	0.1
21.8	26.1	0.1			

* ml. OIB = milliliters of 0.0005N sodium-o-iodosobenzoate
+mV. = positive millivolts
Δ = +mV. increase between two consecutive readings

TABLE 5 A

Amperometric titration of sulfhydryl groups in dairy spread lot 349, using 0.0005N sodium-o-iodosobenzoate

*ml. OIB	+mV.	Δ	ml. OIB	+mV.	Δ
15.0	18.9	-	21.9	26.5	0.1
16.0	18.9	0.0	22.0	26.6	0.1
17.0	18.9	0.0	22.1	26.7	0.1
18.0	18.9	0.0	22.2	26.8	0.1
19.0	18.9	0.0	22.3	26.9	0.1
19.1	18.9	0.0	22.4	27.0	0.1
19.2	18.9	0.0	22.5	27.1	0.1
19.3	18.9	0.0	22.6	27.2	0.1
19.4	19.0	0.1	22.7	27.3	0.1
19.5	19.2	0.2	22.8	27.4	0.1
19.6	19.4	0.2	22.9	27.5	0.1
19.7	19.6	0.2	23.0	27.6	0.1
19.8	20.0	0.4	23.1	27.7	0.1
19.9	20.8	0.8	23.2	27.8	0.1
20.0	21.6	0.8	23.3	27.9	0.1
20.1*	22.8	1.2	23.4	28.0	0.1
20.2	23.5	0.7	23.5	28.1	0.1
20.3	24.2	0.7	23.6	28.2	0.1
20.4	24.8	0.6	23.7	28.3	0.1
20.5	25.2	0.4	23.8	28.4	0.1
20.6	25.4	0.2	23.9	28.5	0.1
20.7	25.5	0.1	24.0	28.6	0.1
20.8	25.6	0.1	24.1	28.7	0.1
20.9	25.7	0.1	24.2	28.8	0.1
21.0	25.7	0.0	24.3	28.9	0.1
21.1	25.8	0.1	24.4	29.0	0.1
21.2	25.9	0.1	24.5	29.1	0.1
21.3	26.0	0.1	24.6	29.2	0.1
21.4	26.1	0.1	24.7	29.3	0.1
21.5	26.1	0.0	24.8	29.4	0.1
21.6	26.2	0.1	24.9	29.5	0.1
21.7	26.3	0.1	25.0	29.6	0.1
21.8	26.4	0.1			

* ml. OIB = milliliters of 0.0005N sodium-o-iodosobenzoate
+mV. = positive millivolts
Δ = +mV. increase between two consecutive readings

TABLE 6 A

Amperometric titration of sulfhydryl groups in dairy spread lot 350, using 0.0005N sodium-o-iodosobenzoate

*ml. OIB	+mV.	Δ	ml. OIB	+mV.	Δ
15.0	18.6	-	21.9	25.2	0.1
16.0	18.6	0.0	22.0	25.3	0.1
17.0	18.6	0.0	22.1	25.4	0.1
18.0	18.6	0.0	22.2	25.5	0.1
19.0	18.6	0.0	22.3	25.6	0.1
19.1	18.6	0.0	22.4	25.8	0.2
19.2	18.7	0.1	22.5	25.9	0.1
19.3	18.9	0.2	22.6	26.0	0.1
19.4	19.1	0.2	22.7	26.1	0.1
19.5	19.3	0.2	22.8	26.2	0.1
19.6	19.5	0.2	22.9	26.3	0.1
19.7	19.7	0.2	23.0	26.4	0.1
19.8	19.9	0.2	23.1	26.5	0.1
19.9	20.2	0.3	23.2	26.6	0.1
20.0	21.0	0.8	23.3	26.7	0.1
20.1*	22.7	1.7	23.4	26.8	0.1
20.2	23.2	0.5	23.5	26.9	0.1
20.3	23.4	0.2	23.6	27.0	0.1
20.4	23.6	0.2	23.7	27.0	0.0
20.5	23.8	0.2	23.8	27.1	0.1
20.6	23.9	0.1	23.9	27.2	0.1
20.7	24.0	0.1	24.0	27.3	0.1
20.8	24.1	0.1	24.1	27.3	0.0
20.9	24.2	0.1	24.2	27.4	0.1
21.0	24.3	0.1	24.3	27.5	0.1
21.1	24.4	0.1	24.4	27.6	0.1
21.2	24.5	0.1	24.5	27.6	0.0
21.3	24.6	0.1	24.6	27.7	0.1
21.4	24.7	0.1	24.7	27.8	0.1
21.5	24.8	0.1	24.8	27.9	0.1
21.6	24.9	0.1	24.9	28.0	0.1
21.7	25.0	0.1	25.0	28.1	0.1
21.8	25.1	0.1			

* ml. OIB = milliliters of 0.0005N sodium-o-iodosobenzoate
+mV. = positive millivolts
Δ = +mV. increase between two consecutive readings

TABLE 7 A

Amperometric titration of sulfhydryl groups in dairy spread lot 351, using 0.0005N sodium-o-iodosobenzoate

*ml. OIB	+mV.	Δ	ml. OIB	+mV.	Δ
15.0	18.0	-	21.9	26.4	0.1
16.0	18.1	0.01	22.0	26.5	0.1
17.0	18.1	0.0	22.1	26.7	0.2
18.0	18.4	0.03	22.2	26.8	0.1
19.0	18.7	0.03	22.3	26.9	0.1
19.1	18.7	0.0	22.4	27.1	0.2
19.2	18.7	0.0	22.5	27.2	0.1
19.3	18.8	0.1	22.6	27.3	0.1
19.4	18.8	0.0	22.7	27.4	0.1
19.5	18.9	0.1	22.8	27.6	0.1
19.6	18.9	0.0	22.9	27.7	0.1
19.7	19.0	0.1	23.0	27.8	0.1
19.8	19.0	0.0	23.1	27.9	0.1
19.9	19.2	0.2	23.2	28.0	0.1
20.0	19.3	0.1	23.3	28.1	0.1
20.1	20.0	0.7	23.4	28.2	0.1
20.2*	21.5	1.5	23.5	28.3	0.1
20.3	22.7	1.2	23.6	28.3	0.0
20.4	23.5	0.8	23.7	28.3	0.0
20.5	23.8	0.3	23.8	38.3	0.0
20.6	24.1	0.3	23.9	28.4	0.1
20.7	24.3	0.2	24.0	28.4	0.0
20.8	24.6	0.3	24.1	28.4	0.0
20.9	24.8	0.2	24.2	28.5	0.1
21.0	25.0	0.2	24.3	28.5	0.0
21.1	25.2	0.2	24.4	28.5	0.0
21.2	25.4	0.2	24.5	28.6	0.1
21.3	25.6	0.2	24.6	28.6	0.0
21.4	25.8	0.2	24.7	28.7	0.1
21.5	26.0	0.2	24.8	28.7	0.0
21.6	26.1	0.1	24.9	28.8	0.1
21.7	26.2	0.1	25.0	28.8	0.0
21.8	26.3	0.1			

* ml. OIB = milliliters of 0.0005N sodium-o-iodosobenzoate
+mV. = positive millivolts
Δ = +mV. increase between two consecutive readings

TABLE 8 A

Amperometric titration of sulfhydryl groups in dairy spread lot 352, using 0.0005N sodium-o-iodosobenzoate

*ml. OIB	+mV.	Δ	ml. OIB	+mV.	Δ
15.0	18.8	-	21.9	24.8	0.0
16.0	18.8	0.0	22.0	24.9	0.1
17.0	18.8	0.0	22.1	25.0	0.1
18.0	19.2	0.04	22.2	25.1	0.1
19.0	19.9	0.07	22.3	25.2	0.1
19.1	20.0	0.1	22.4	25.3	0.1
19.2	20.1	0.1	22.5	25.4	0.1
19.3	20.2	0.1	22.6	25.5	0.1
19.4	20.3	0.1	22.7	25.6	0.1
19.5	20.6	0.3	22.8	25.7	0.1
19.6	20.9	0.3	22.9	25.8	0.1
19.7*	22.8	1.9	23.0	25.9	0.1
19.8	23.5	0.7	23.1	26.0	0.1
19.9	23.7	0.2	23.2	26.1	0.1
20.0	23.8	0.1	23.3	26.2	0.1
20.1	23.9	0.1	23.4	26.3	0.1
20.2	24.0	0.1	23.5	26.4	0.1
20.3	24.0	0.0	23.6	26.5	0.1
20.4	24.1	0.1	23.7	26.6	0.1
20.5	24.1	0.0	23.8	26.7	0.1
20.6	24.2	0.1	23.9	26.8	0.1
20.7	24.2	0.0	24.0	26.9	0.1
20.8	24.3	0.1	24.1	27.0	0.1
20.9	24.3	0.0	24.2	27.1	0.1
21.0	24.4	0.1	24.3	27.2	0.1
21.1	24.4	0.0	24.4	27.3	0.1
21.2	24.5	0.1	24.5	27.4	0.1
21.3	24.5	0.0	24.6	27.5	0.1
21.4	24.6	0.1	24.7	27.6	0.1
21.5	24.6	0.0	24.8	27.7	0.1
21.6	24.7	0.1	24.9	27.8	0.1
21.7	24.7	0.0	25.0	27.9	0.1
21.8	24.8	0.1			

* ml. OIB = milliliters of 0.0005N sodium-o-iodosobenzoate
+mV. = positive millivolts
Δ = +mV. increase between two consecutive readings

TABLE 9 A

Amperometric titration of sulfhydryl groups in dairy spread lot 353, using 0.0005N sodium-o-iodosobenzoate

*ml. OIB	+mV.	Δ	ml. OIB	+mV.	Δ
15.0	17.2	-	21.9	19.6	0.1
16.0	17.2	0.0	22.0	19.7	0.1
17.0	17.2	0.0	22.1	19.7	0.0
18.0	17.3	0.01	22.2	19.7	0.0
19.0	17.4	0.01	22.3	19.7	0.0
19.1	17.4	0.0	22.4	19.7	0.0
19.2	17.4	0.0	22.5	19.7	0.0
19.3	17.4	0.0	22.6	19.7	0.0
19.4	17.4	0.0	22.7	19.8	0.1
19.5	17.4	0.0	22.8	19.8	0.0
19.6	17.4	0.0	22.9	19.8	0.0
19.7	17.4	0.0	23.0	19.8	0.0
19.8	17.4	0.0	23.1	19.8	0.0
19.9	17.4	0.0	23.2	19.8	0.0
20.0	17.4	0.0	23.3	19.8	0.0
20.1	17.4	0.0	23.4	19.8	0.0
20.2	17.5	0.1	23.5	19.8	0.0
20.3	17.5	0.0	23.6	19.8	0.0
20.4	17.5	0.0	23.7	19.8	0.0
20.5	17.6	0.1	23.8	19.8	0.0
20.6*	18.1	0.5	23.9	19.8	0.0
20.7	18.3	0.2	24.0	19.8	0.0
20.8	18.4	0.1	24.1	19.8	0.0
20.9	18.5	0.1	24.2	19.9	0.1
21.0	18.6	0.1	24.3	19.9	0.0
21.1	18.7	0.1	24.4	20.0	0.1
21.2	18.9	0.2	24.5	20.0	0.0
21.3	19.0	0.1	24.6	20.1	0.1
21.4	19.1	0.1	24.7	20.2	0.1
21.5	19.2	0.1	24.8	20.2	0.0
21.6	19.3	0.1	24.9	20.2	0.0
21.7	19.4	0.1	25.0	20.2	0.0
21.8	19.5	0.1			

* ml. OIB = milliliters of 0.0005N sodium-o-iodosobenzoate
+mV. = positive millivolts
Δ = +mV. increase between two consecutive readings

TABLE 10 A

Amperometric titration of sulfhydryl groups in dairy spread lot 354, using 0.0005N sodium-o-iodosobenzoate

*ml. OIB	+mV.	Δ	ml. OIB	+mV.	Δ
15.0	18.2	-	21.9	27.9	0.1
16.0	18.4	0.02	22.0	28.0	0.1
17.0	18.7	0.03	22.1	28.1	0.1
18.0	18.9	0.02	22.2	28.2	0.1
19.0	19.1	0.02	22.3	28.2	0.0
19.1	19.1	0.0	22.4	28.3	0.1
19.2	19.2	0.1	22.5	28.4	0.1
19.3	19.4	0.2	22.6	28.4	0.0
19.4*	21.8	2.4	22.7	28.5	0.1
19.5	22.6	0.8	22.8	28.5	0.0
19.6	23.0	0.4	22.9	28.6	0.1
19.7	23.5	0.5	23.0	28.7	0.1
19.8	24.0	0.5	23.1	28.7	0.0
19.9	24.5	0.5	23.2	28.8	0.1
20.0	24.9	0.4	23.3	28.8	0.0
20.1	25.2	0.3	23.4	28.9	0.1
20.2	25.5	0.3	23.5	28.9	0.0
20.3	25.7	0.2	23.6	29.0	0.1
20.4	26.0	0.3	23.7	29.0	0.0
20.5	26.3	0.3	23.8	29.1	0.1
20.6	26.5	0.2	23.9	29.1	0.0
20.7	26.7	0.2	24.0	29.2	0.1
20.8	26.8	0.1	24.1	29.2	0.0
20.9	26.9	0.1	24.2	29.3	0.1
21.0	27.0	0.1	24.3	29.3	0.0
21.1	27.1	0.1	24.4	29.3	0.0
21.2	27.2	0.1	24.5	29.4	0.1
21.3	27.3	0.1	24.6	29.4	0.0
21.4	27.4	0.1	24.7	29.4	0.0
21.5	27.5	0.1	24.8	29.5	0.1
21.6	27.6	0.1	24.9	29.5	0.0
21.7	27.7	0.1	25.0	29.6	0.1
21.8	27.8	0.1			

* ml. OIB = milliliters of 0.0005N sodium-o-iodosobenzoate
+mV. = positive millivolts
Δ = +mV. increase between two consecutive readings

TABLE 11 A

Amperometric titration of sulfhydryl groups in dairy spread lot 355, using 0.0005N sodium-o-iodosobenzoate

*ml. OIB	+mV.	Δ	ml. OIB	+mV.	Δ
15.0	18.2	-	21.9	24.6	0.1
16.0	18.2	0.0	22.0	24.7	0.1
17.0	18.3	0.01	22.1	24.8	0.1
18.0	18.4	0.01	22.2	24.9	0.1
19.0	18.5	0.01	22.3	25.0	0.1
19.1	18.6	0.1	22.4	25.1	0.1
19.2	18.8	0.2	22.5	25.2	0.1
19.3	18.9	0.1	22.6	25.3	0.1
19.4	19.1	0.2	22.7	25.4	0.1
19.5	19.2	0.1	22.8	25.5	0.1
19.6	19.4	0.2	22.9	25.6	0.1
19.7	19.5	0.1	23.0	25.7	0.1
19.8	19.7	0.2	23.1	25.8	0.1
19.9	19.8	0.1	23.2	25.9	0.1
20.0	19.9	0.1	23.3	26.0	0.1
20.1	19.9	0.0	23.4	26.1	0.1
20.2	19.9	0.0	23.5	26.2	0.1
20.3	19.9	0.0	23.6	26.3	0.1
20.4	19.9	0.0	23.7	26.4	0.1
20.5	20.0	0.1	23.8	26.5	0.1
20.6	20.6	0.6	23.9	26.6	0.1
20.7*	21.5	0.9	24.0	26.7	0.1
20.8	22.0	0.5	24.1	26.8	0.1
20.9	22.4	0.4	24.2	27.0	0.2
21.0	22.8	0.4	24.3	27.1	0.1
21.1	23.1	0.3	24.4	27.2	0.1
21.2	23.4	0.3	24.5	27.3	0.1
21.3	23.6	0.2	24.6	27.5	0.2
21.4	23.8	0.2	24.7	27.6	0.1
21.5	24.0	0.2	24.8	27.7	0.1
21.6	24.2	0.2	24.9	27.8	0.1
21.7	24.4	0.2	25.0	27.9	0.1
21.8	24.5	0.1			

* ml. OIB = milliliters of 0.0005N sodium-o-iodosobenzoate
+mV. = positive millivolts
Δ = +mV. increase between two consecutive readings